STABILITY FUNCTION OF PLASMID ColK

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by

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This thesis is dedicated to my mother, wife and family for all their loving support and prayers over the last two years.

CONTENTS

ABBREVIATIONS

i. Chemicals

Ac	acetate
APS	ammonium persulphate
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
DS	Denhardts solution
DTT	dithiothreitol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphoshate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
dH_2O	distilled water
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
EtBr	ethidium bromide
EtOH	ethanol
oc	covalently closed relaxed DNA
RNA	ribonucleic acid
sc	supercoiled DNA
SDS	sodium dodecylsulphate
TEMED	N,N,N',N'-tetramethyl ethylenediamine
Tris	tris(hydroxymethyl)aminomethane

ii. **Antibiotic**

Ap	Ampicillin
Str	Streptomycin(chromosomal resistance)
Tc	Tetracycline
SmSu	streptomycin and sulfonamide

iii. **Phenotype**

Ika^r	resistance to colicin k
Iea^r	" " " E
Cea	Colicin E
Cka	Colicin K
cer	recombination in ColE1
ckr	recombination in ColK
X^r	resistance to X
X^s	sensitivity to X
Ori	origin of replication
res	resolution site
Rec^+	recombination proficient

iv. **Measurements**

mA	milliamps
bp	basepair

kb	kilobasepair
^{o}C	degrees centigrade
cpm	counts per minute
hr	hour
g	centrifugal force equal to gravitational acceleration
g	gramme
mg	milligramme
μg	microgramme
ng	nanogramme
l	litre
ml	millilitre
μl	microlitre
M	Molar (moles per litre)
mM	millimolar
μM	micromolar
cm	centimetre
mm	millimetre
min	minute
pH	acidity [negative $\log_{10}$(Molar concentration H^{+} ions)]
sec	second
V	Volts
w/v	weight per volume
v/v	volume per volume
W	Watts

v. Miscellaneous

Fig.	figure
Tn	transposon
wt	wild-type

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SUMMARY

ColK is a multicopy naturally occurring plasmid, that is very stably maintained in bacterial host cells. This work shows that the plasmid encodes a stability determinant designated *ckr*, that is involved in promoting site-specific recombination in multimeric plasmids, efficiently converting them to monomers. This apparently ensures stability by maximizing the number of segregating units at cell division.

ckr has been located to a 0.37 kb fragment in ColK, its DNA sequence is highly homologous with that of *cer* (stability determinant of ColE1, a related plasmid to ColK). Recombination has been found to occur both between directly repeated *cer* copies and a direct repeat of one copy of *cer* and one of *ckr* and results in the deletion of the internal fragment between them.

Inversion has been shown to occur between two *cer* copies in inverted repeat and in a substrate containing a copy of *cer* and a copy of *ckr* in inverted repeat. Both deletion and inversion are highly efficient.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Bacterial plasmids are circular double stranded DNA molecules that are physically separated from the bacterial chromosome. They are stably maintained and capable of autonomous replication.

Plasmids have been found in representitive members of all bacteria, Gram-positive, Gram-negative and Archaeobacteria (for reviews see Broda, 1979). Some plasmids carry determinants for drug resistance, and many other plasmids confer resistance to metal ions and organometallic compounds. Another group of plasmid-born functions are those for degradation of semi-exotic organic compounds. For example, TOL plasmids. Plasmid-born determinants to utilize lactose have been described from a wide range of enterobacterial species. Determinants for microbial pathogenicity are often encoded by plasmids, for example, in some cases the ability to cause diseases depends upon the presence of enterotoxin-producing (Ent) plasmid have been reported in the strain of *E. coli*. Plasmid-like systems have been found in some simple eukaryotes (e.g. plasmid termed the 2 micron circle DNA in most strains of *Saccharomyces cerevisiae*, (Broach, 1982).

Naturally occurring plasmids in enteric bacteria can be divided into two groups on the basis of their size: Group I, small plasmids of molecular weight less than 15 kb, and group II, large plasmids with molecular weights of between 30 and 500 kb. These two groups

generally differ markedly in their copy numbers per cell, group I plasmids being multicopy (20-200 copies per cell) and group II plasmids being present at only one or two copies per chromosome.

Only now it is being recognized how common plasmids are in bacteria. This realization comes from the development and application of methods for isolating plasmid DNA. Relating phenotypes to such plasmids will often depend upon developing transformation methods for the particular systems. The current view is that the role of plasmids in nature is to carry optional functions. This view is partly held because many plasmids are indeed dispensable and partly because of the intuitive feeling that essential functions are best carried by the chromosome.

Genetic methods showed that many plasmids appeared to be related in their transfer properties and replication. Thus, F is related to R1, R6, R100 and ColV (Sharp et al, 1973) and also a typical Ent plasmid (Santos et al, 1975). Different types of plasmids therefore form an interconnected population.

There are a number of examples of independently isolated plasmids (e.g. R factors, Col factors, Ent plasmids) showing indistinguishable properties of part or the whole plasmid, suggesting a common origin. The following are examples: (1) Similar TOL plasmids were found in independently isolated organisms. (2) At least some of the known transposons are widely distributed. When ampicillin-resistant Haemophilus influenzae type b and gonococcal strains appeared, the resistance function was homologous with that of one in E. coli. (3) After a number of years of use of chloramphenicol, a set of related R factors appeared in strains of Salmonella typhimurium. Analogous

cases have been reported among the SmSu plasmids (Grinter and Barth, 1976) and R plasmids of strains of Shigella (Crosa et al, 1977). (4) Some of the Ent plasmids form an homogenous group.

It may be concluded that, in spite of the very widespread occurrence of drug resistance and other functions on plasmids, a large proportion of the reported cases are probably due to a few originating events.

Under laboratory conditions plasmid characterized in a particular host can often be established in very different strains. Examples are the staphylococcal plasmids introduced in Bacillus subtilis (Ehrlich, 1977) and transfer of the following into E. coli: TOL from Ps putida (Benson and Shapiro, 1978); Ti from A. tumefaciens (Holsters et al, 1978); R plasmids from bacteroides ochraccus, and the P1 group plasmid of Pseudomonas (for reviews on the ubiquity of plasmids see, Broda, 1979, and Sherratt, 1982).

1.2 Phenotypic Properties of Plasmids

1.2.1 Multiple Drug Resistance

Ever since the introduction of antibiotics as a means of controlling infectious disease, bacterial strains have emerged that are resistant to a variety of chemotherapeutic agents. The examination of the mechanism of resistance in resistant strains which have been isolated from nature has revealed a variety of ways in which microorganisms can survive in the presence of antibiotics. According to the tenets of microbial genetics, mutant strains should acquire resistance to only one antibiotic at a time, and organisms resistant to several drugs should arise in nature only by the accumulation of successive mutations. It is therefore quite extraordinary that there has been a dramatic increase in the frequency of occurrence of Enterobacteriaceae with multiple drug resistance in essentially all countries in which the problem has been examined.

In general, the multiple drug resistance of these microorganisms does not seem to have arisen in a series of discrete steps; but rather, resistance to all of the drugs appears to have been acquired simultaneously. Genetic analysis has revealed that multiple drug resistance is specified by an extrachromosomal element which is referred to as a drug-resistance factor or R factor. Over the past two decades, R factors have been studied extensively throughout the world.

The first R factors were detected in Japan in the 1950's during an outbreak of bacillary dysentry (Watanabe, 1963). Since that time, extensive epidemiological studies that were carried out have shown that in numerous clinical situations, R factors are the agents responsible for resistance to antibiotics in Enterobacteriaceae. The occurrence of R^+ strains is not simply restricted to hospital outbreaks; R factors are present in domestic animals (Anderson, 1968) and fish particularly when antibiotics are used as routine diet additives.

Resistances to at least ten antibiotics are specified by R factors, and it seems likely that additional resistance determinants will be found in the future. No R factor that has been isolated from nature harbours all of these resistance determinants simultaneously. There appears to be no obvious pattern of development of antibiotic resistance, although the number of resistance markers can increase as a result of exposure of the organism to additional antibiotics (Datta 1975).

It has been shown that enteric bacteria that carried R factors were present before the advent of wide spread antibiotic therapy (Smith, 1967; Gardner et al, 1969).

Numerous classifications of R factors are possible, and different R factor isolates have characteristic groupings of resistance markers. The R factors are often given different designations by different groups of workers for example, NR1, R100 and R222 are one and the same, many of the R factors that were isolated in different parts of the world are differently named, may in fact be identical.

Several groups of Japanese genetists have shown that R factors are extrachromosomal genetic elements that are capable of autonomous replication; that is, they replicate independently of the host chromosome. Most of the initial studies were on the R factor NR1, which had originally been isolated from nature in a strain of Shigella. In general, most of the initial results obtained with NR1 appear to be characteristic of other R factors, which have not been studied in as much detail. The R factors can be transferred by bacterial conjugation to essentially all members of the Enterobacteriaceae (Nakaya et al, 1960). The transduction of multiple drug resistance by appropriate temperate phages has been observed in E. coli, Shigella flexneri, several Salmonella species, and Proteus mirabilis (Watanabe et al, 1961; Harada et al, 1963).

The transfer of multiple drug resistance, either by bacterial conjugation or transduction, takes place independently of the transfer of host chromosome genes.

1.2.2 Bacteriocins

In 1952, Gratia demonstrated that some bacteria released agents (bateriocins) into the surrounding medium that killed other bacteria, but to which other individuals of the producing strain were resistant. This killing is observed on agar plates as clear zones on lawns of sensitive bacteria, each zone originating from a single bacteriocinogenic bacterium, normally only a small proportion of the

cells of such a population are actively producing such bacteriocin. However, agents that include ultraviolet light induce the synthesis of some bacteriocins. Bacteriocins have been described from a wide range of Gram-positive and Gram-negative bacteria (Reeves, 1972). Bacteriocins are given names that indicate their origins: examples are megacins from Bacillus megaterium, subtilicins from Bacillus subtilis and colicins from E. coli. They may have evolutionary relations with other structures, such as pili, toxins and phage proteins. The biological role of bacteriocins seems to be to give the carrier strain (and the population of plasmids that these cells carry) a selective advantage over other closely related strains, by killing them. Although the minority of cells that actually produce the bacteriocin are killed themselves, the population as a whole can then prosper.

All colicins studied so far are proteins specified by plasmids. Colicins are classified on the basis of their activity spectra against other strains. Such analysis is sometimes complicated by the presence of more than one Col factor in a cell. An alternative method of classification has been by determining the activity of colicins against a set of derivatives of an originally sensitive strain, each derivative being resistant to one of a series of colicins. This approach constitute the basis of current colicin classification.

Col factors (i.e. the plasmids) fall into the same two classes as do R factors. Some are large, about 60 M dal, conjugative, and present in only a few copies, while others are about 5 M dal, non-conjugative and present in many copies.

Several colicin receptors have been identified as proteins in the outer membrane. In many but not all instances resistance is due to alteration or loss of these receptors. There are three ways in which colicins are known to kill cells. (1) Some such as E1 and K, appear to act on the cytoplasmic membrane, and to uncouple energy-dependent processes. The loss of potassium ions from colicin-treated cells has been noted. (2) Colicin E2 brings about the degradation of DNA and inhibition of cell division (Nomura, 1963), presumably after entering the cell. Purified colicin E2 is inactive against DNA until a tightly bound immunity protein is removed (Schaller and Nomura, 1976). (3) Colicin E3 prevents protein synthesis by cleaving the 16-S ribosomal RNA (Bowman et al, 1971). Thus it is a nuclease acting on RNA rather than DNA.

1.2.3 Other Plasmid Phenotypes

Many R factors (and other plasmids) confer resistance to metal ions and/or organometallic compounds (Summers and Jacoby, 1977; Clark et al, 1977; and Dean et al, 1977). It has been observed that more of a series of Ps. aeruginosa clinical isolates were resistant to Hg^{2+} than to any of a range of antibiotics.

R plasmids can also determine altered sensitivity to radiation and mutagens (Smith et al, 1976; and Jacoby and Sutton, 1977).

There are plasmids that contribute directly to virulence. In some *E. coli* strains the production of enterotoxins depends on the presence of Ent plasmids. The symptoms of diseases caused by Ent plasmid-containing *E. coli* are similar to those of cholera and can be almost as acute (Finkelstein *et al*, 1976).

The presence of Ent plasmid in *E. coli* is not sufficient to cause diarrhoea in piglets. It was observed that another plasmid, specifying the (capsular) K88 antigen, is also required (Smith and Linggood, 1971).

It has been known that pseudomonads are able to utilize a wide range of organic compounds as carbon and energy sources. Some of the metabolic functions have been mapped at chromosomal loci but it emerges that some are catabolized by plasmid-specified enzymes. At least in the case of the TOL (toluate), plasmid the plasmid carries a whole array of functionally related genes (Worsey and Williams, 1975).

It has been recognized that there are similarities between temperate phages and plasmids in bacteria. Since some viruses are tumorogenic it was possible that viruses and/or plasmids carried by bacteria could also cause tumors. 'Ti' plasmids of *A. tumefaciens* have this property (Holsters *et al*, 1978).

It is clear now that genes determining nodulation, nitrogen fixation and host-range are plasmid-born in at least some *Rizobium* species.

1.3 Plasmid Maintenance

1.3.1 Replication of ColE1-like Plasmids

Because ColE1 is a related plasmid to ColK plasmid (the subject of this thesis), I will discuss its replication which has been intensively studied. ColE1 is a member of a group of small plasmids that can replicate in the absence of de novo protein synthesis (Staudenbauer, 1978; Veltkamp and Stuitje, 1981). ColE1 does not require any plasmid encoded proteins for replication in vivo, and the necessary host proteins are stable.

For replication all the members of the ColE1 group of replicons require the host encoded enzymes DNA polymerase I (Kingsbury and Helinski, 1973) (product of polA gene) and DNA-dependent RNA polymerase (Cozzarelli et al, 1968; Crosa et al, 1975; Staudenbauer, 1975) as well as DNA polymerase III (the product of dnaE). The products of E. coli dnaB, C, G and Z are also required.

Replication of ColE1 is unidirectional from a specific origin site in vivo, as shown by electron microscopy (Inselburg, 1974; Lovett et al, 1974; Tomizawa et al, 1974).

Rifamycin-sensitive RNA synthesis is required for ColE1 replication (Blair et al, 1972; Clewell et al , 1972; Sakakibara et al, 1974).

DNA replication starts at any of the three consecutive bases defining the origin of replication (Tomizawa et al, 1977). The RNA primer promoter is located about 555 bases upstream of the replication origin. In the absence of DNA initiation, several sizes of transcripts that continue through the replication origin are synthesized (Fig. 1.1). However, some of the nascent transcripts hybridize with their template DNA near the origin. These RNA-DNA hybrids serve as the substrate for RNase H which cleaves the hybridized preprimer RNA to produce the RNA primer (RNA IV) (Itoh & Tomizawa , 1980) Deoxynucleotides are added directly to the primer by the host enzyme DNA polymerase I. The formation of a stable hybrid between the RNA preprimer and DNA is thus critical for initiation of DNA replication. The formation of the critical preprimer RNA-DNA hybrid is under negative control (Hashimoto-Gotoh and Inselberg, 1979; Shepard et al, 1979) by a small RNA molecule called RNA I, whose presence leads to continuation of transcription through the replication origin instead of processing of the preprimer transcript by RNase H (Tomizawa et al, 1981). RNA I is transcribed from the region shown genetically to be involved in copy number control (Conard et al, 1979; Heffron et al, 1978; Moser et al, 1983). It is about 100 nucleotides long (Levin and Rupp, 1978; Oka et al, 1979), starts 400 to 480 bases upstream the origin, and ends near the start of the primer transcript (see Fig. 1.1). Thus the same DNA region that encodes primer RNA is used in the opposite direction to transcribe RNA I. From their sequences, it can be deduced that both the inhibitory RNA I and the preprimer RNA II can form three stem-and-loop structures.

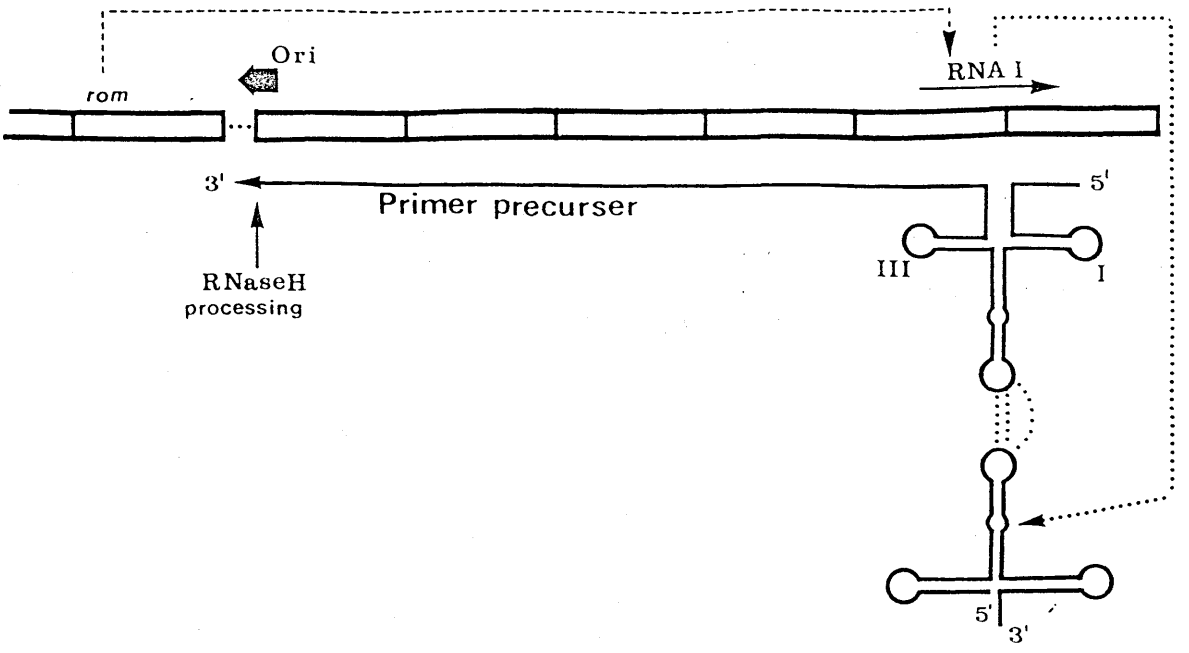


Fig 1.1 Replication region of ColE1

The direction of DNA replication is indicated by the dark arrow, which starts from "ori". The open bar represents the DNA, and the vertical bars indicate distances of 100 nucleotides. The transcripts are indicated as arrows above the DNA.

This figure is derived from one by Lacenta and Cesareni, 1983.

In vitro, RNA I inhibits formation of the primer (Itoh and Tomizawa, 1980) probably by inhibition of the formation of DNA-RNA hybrid between the primer precursor and its DNA template.

A transacting regulatory element was mapped to a region bounded by two HaeII restriction sites 806 and 184 nucleotides downstream the replication origin of pMB1 (Twigg and Sherratt, 1980). This region is located between the replication origin and the mobilization genes of ColE1 and is not essential for plasmid replication. Later it was shown that this region encodes a 63 a.a polypeptide trans-acting element that inhibits transcription initiated at the primer promoter. The function defined by this region was originally named *rop*, for repressor of promoter (Cesareni *et al*, 1982) though Tomizawa now proposes *rom*, for repressor of modulator. It was proposed that this polypeptide is the *rom* gene product and it regulates plasmid DNA replication by enhancing the action of RNA I replication repressor (Scott, 1984). The deletion of *rom* results in a 7-fold increase in copy number (Twigg and Sherratt, 1980) and also in a decrease in plasmid stability (C. Jones, pers. comm.).

The fact that RNAI must be able to act *in trans* on preprimer to control copy number, also means that the specificity of the RNAI/preprimer reaction provides a molecular explanation of plasmid incompatibility in ColE1 group of plasmids. Incompatibility is defined as the inability of two (or more) plasmids to coexist stably in the same cell line. Incompatible plasmids share the same replication control system. Plasmids that inhabit a particular

bacterial host can be divided into groups (Inc groups) by their incompatibility relationships, and this has formed a basis for plasmid classification.

Moreover, it is generally true that conjugative plasmids within an Inc group have identical or closely related transfer genes and their conjugative pili are morphologically and serologically similar, whereas plasmids of different groups have different conjugative genes and unrelated pili (Bradley, 1980).

1.3.2 Plasmid Stability

Most of the naturally occurring plasmids are very stably maintained in growing populations of host bacteria. Whereas most of the multicopy cloning vectors are lost at significant frequencies. This indicates that in addition to a stringent system for replication control, plasmids must encode functions that ensure that each daughter cell receives a plasmid copy.

We can think of two theories to explain partition of plasmid at cell division partition of plasmids appears to occur in two different ways The first, involves active distribution or partition of some or all of the plasmids. The other way is a random one, in which the plasmids are partitioned non actively. Actively partitioned plasmids should be extremely stable, while random partition will result in the appearance of plasmid-free cells at frequencies related to the copy number of the plasmid in the dividing cell. If low copy number plasmids (which have

copy numbers of one or two plasmids per dividing cell) are randomly distributed, they will be lost at very high rates, approaching about 50% per cell division event. Most of the evidence on partition of low copy number plasmids is consistent with the active partition theory. Two models for active partitioning of low copy number plasmids were proposed. Equipartitioning and Pair Site Partitioning, in the former each daughter cell receives the same number of plasmid copies at cell division (or, for odd numbers, one of the daughters receives one copy more than the other), and in the latter, each cell gets one copy by the partitioning mechanism, whereas the rest are randomly partitioned. A plasmid which has a copy number of 3-4 copies per cell, at cell division, plasmid-free cells were estimated to appear at a frequency of $1.5 \times 10^{-3} - 8 \times 10^{-5}$ for equipartitioning, and $6 \times 10^{-3} - 6 \times 10^{-4}$ for pair site partitioning (Nordstrom and Agaard-Hansen, 1984). This means a very high degree of stability. However, most of the results on stability of naturally occurring low copy number plasmid appear to be consistent with these calculation. And most of the data on mult^{i}copy cloning vectors (derivatives of naturally occurring plasmids) seem to be in agreement with the random distribution model (Novick et al, 1975). The presence of a function responsible for the active partition (par or stb) has been found in plasmids R1 (Nordsrom et al, 1980), F (Ogura and hiraga, 1983), NR1 (Miki et al, 1980), pSC101 (Meacock and Cohen, 1980), P1, P7 (Austin and Abeles, 1983), pTAR (Gallie et al, 1984), pCU1 (Konarska-Kozlowska and Iyer, 1984) and RK2 (Thomas et al, 1984).

In pSC101, studies on partition have shown that three discrete segments of DNA within the *par* region of pSC101 are involved in partitioning of the plasmid to daughter cells, the three segments are termed a, b and a'. The position and the dyad symmetry of these segments are such that hairpin-loop structures potentially can be formed by pairing of the middle segment (b) with either of the two lateral segments (a or a'). Deletions involving any two of the three segments yield a plasmid that is not stably maintained and which has par^- phenotype. Deletion of only one of the lateral segments (a or a') yields a plasmid that is phenotypically is Cmp^- (competition negative); although stable in a cell lacking another plasmid, it is unable to compete on equal terms with a coexisting wild-type plasmid having an identical replication system. Deletion of all three segments (a, b and a') yields plasmids that show the super-par^- phenotype; in the absence of selection, these are lost at a much faster rate than would be expected for random distribution.

These results suggested a model in which the *par* function enables the nonreplicating plasmid in the dividing cell to be counted as individual molecules, and distributed actively and evenly to daughter cells. In the total absence of *par* function (super-par^-), the plasmids are presumed to form a multicopy cluster which behaves like a single unit at cell division. The simple par^- phenotype represents a lesser degree of loss of *par* function; there is some capacity for counting although not evenly partitioning. Presumably, the presence of one intact segment in a simple par^- mutants is sufficient to impart some residual *par* function (Tucker *et al*, 1984).

The pSC101 *par* fragment has been shown to stabilize partition-defective derivatives of pSC101 replicon when present in *cis*, but not when located in *trans* on a coexisting plasmid (Meacock and Cohen, 1980), and also to increase the stability of unrelated plasmids derived from the p15A and R1 replicons (Meacock and Cohen, 1980; Norstrom *et al*, 1980a)

It has been demonstrated that the F plasmids contain a segment required for reliable partitioning of the plasmid at cell division. This segment is located outside the segment essential for replication, and that the former segment has three functional regions essential for partition. By trans-complementing analyses, it was found that two of the three regions, termed *sopA* and *sopB*, specify trans-acting functions and that the third region termed *sopC*, acts only a *cis* position (Ogura and Hiraga, 1983). The *sopA* and *sopB* genes seem to correspond to the region encoding proteins with molecular weight of 41 to 44 kd and 36 to 37 kd, respectively (Wehlmann and Eichenlaub, 1983; Komai *et al*, 1982). It was therefore, proposed that the proteins encoded by *sopA* and *sopB* act in *trans* to perform partition of plasmid. Recently, it was found that the 37 kd protein encoded by *sopB* binds to a unique DNA region corresponding to *sopC* region. And it also has been shown that two proteins (75 and 33 kd), which are supposed to be encoded by the host chromosome, bind to the DNA segment to which the *sopB* protein binds (Ogura and Hiraga, 1983).

It was proposed that, the *sopC* region on the plasmid molecule seems to interact with the cellular component consisting of the *sopB* protein (37 kd) and at least two host proteins (75 and 33 kd). The *sopB* protein may act to associate plasmid DNA molecules with the

cellular components through its binding to the sopC region. The product of sopA may interact with the sopC region or indirectly with the cellular component associated with the sopC region.

Full stability of the R1 plasmid has been found to require two different regions of DNA from R1 itself, these two regions are termed par A and par B. Although the stability exerted by each of these regions when inserted into mini-R1 plasmids was not complete, as observed for both regions, the loss rate of mini-R1 par A^+ or mini-R1 par B^+ plasmids was 100-fold less than unstable plasmids (Gerdew et al, 1985).

The par B^+ fragment seems much more efficient in stabilizing unrelated plasmids than does the par A^+ fragment. In contrast, they both stabilize mini-R1 to the same extent. This indicates that par A^+ fragment requires a region of plasmid R1.

The unit-copy P1 plasmid has been shown to contain a par region which consists of approximately 2.5×10^3 base pair segment of DNA of which the terminal kd contains the plasmid incompatibility determinant inc B. Partition defective mutants were complemented by the wild-type par region in trans. The complementing activity was shown to be an M_r 44,000 protein encoded by the end of the par region distal to inc B. The sequence of inc B was found to be essential in cis to the plasmid in order that the plasmid be receptive to the par protein activity, and that the protein-DNA complex constitutes an essential element for association of the plasmid with the putative host cell partition apparatus.

In general very little is known about the mechanism that controls partitioning. However, there seems to be a randomization step involved resulting in a weak incompatibility between two (compatible) plasmids that have the same par function (Nordstrom et al, 1980). This could indicate a competition for a site. This site could be connected with the so-called folded chromosome (Worcel and Burgi, 1974), since many plasmids co-sediment with the folded chromosome (Kline et al, 1976). However, a more likely correlation might be involvement of membrane attachment. The Par^+ region of plasmids R1 and pSC101 was shown to cause a specific binding to membrane material with a density corresponding to that of the outer membrane of the host bacteria (Gustafsson et al, 1983).

There are indications of a correlation between the presence of a plasmid in a cell and fidelity of cell division; copy mutants of plasmid R1 cause failure of cell division in a significant fraction of the host bacteria (Engberg et al, 1975). There is evidence for a coupling between replication of certain plasmids and cell division (Rosner et al, 1968); if these plasmids do not replicate the cell cannot divide; recA cells, however, do divide under such circumstances (MacQueen and Donachie, 1977).

At the stage where plasmid-free cells start to appear after a comparative shift of a population carrying a rep(ts) derivative of plasmid F, a significant proportion of the cells become elongated, indicating an effect on cell division in cells containing only one plasmid copy, suggested that initiation of replication sets up a block of cell division that can be relieved only by completion of the replication of that replicon. (Donachie, 1969, 1974). It has been

proposed that F plasmid encodes a function designated ccd (control of cell division) which is able to block cell division when the plasmid copy number becomes very low (Ogura and Hiraga 1983)

It is possible that this observed effect on cell division is mediated by the par function rather than the rep function. Plasmid R1 for example, is extremely stably inherited suggesting that cells are not allowed to divide until they contain at least two copies of R1. However, there is no such limitation if the cells contain par$^-$ derivatives of R1 (Nordstrom et al, 1980).

The copy number of multicopy plasmids is usually sufficient that they need not have an active partition system; random segregation would be sufficient for stable maintenance. No active partition loci have been documented for ColE1 related plasmids and the available evidence is consistent with random partition (Summers and Sherratt, 1984). Nevertheless such plasmids do have stability determinants and their properties are described below.

1.3.3 Generalized Recombination and Plasmid Stability

E. coli like all the naturally occurring bacterial strains, shows the capacity to promote generalized recombination between homologous DNA sequences. There are some chromosomal functions which mediate recombination of plasmid replicons.

Generalized recombination in E. coli has been demonstrated to occur through three pathways; recBC, recE and recF. The recBC pathway

is the major pathway for chromosomal recombination in E. coli wild-type cells. This pathway depends on the activity of the recA and recB recC gene products (Clark, 1973, 1980). Mutations in the recB recC gene can be indirectly suppressed by two mutations. The sbcA mutation, which activates the recE pathway by derepressing the synthesis of the ATP-independent exonuclease VIII (Barbour et al, 1970), and by the sbcB mutation which activates the recF pathway by inactivating exonuclease I (Kushner et al, 1972).

Intermolecular recombination in plasmid replicons leads to the formation of multimers. Multimerization has been demonstrated to occur at high frequency in recBC$^-$ (Bedbrook and Ausubel), and rec$^+$ hosts. If the copy number control system keeps a constant number of plasmid replication origins in the dividing cell, multimerization will decrease the number of plasmid molecules and since a multimer behaves like a single segregation unit, the number of the segregation units will be much less than the number of the segregation units when the plasmid molecules are in monomeric form. With low copy number plasmids multimerization will prevent active partition whereas with high copy number plasmid multimerization will decrease the number of segregating units available for random partition. This is capable of creating a sub-population of cells with low plasmid copy number and these cells will generate plasmid-free cells at high frequencies. Also if the initiation of plasmid replication starts at any replication origin within the cell, multimers will have a replication advantage over the monomeric molecule because multimers contain more replication origins than monomers, leading to a clonal inheritance of multimers.

Stability of multicopy plasmids has been found to be decreased by multimerization (Summers and Sherratt, 1984). For example, these plasmids were least stable in a recBC sbcA strain (recE pathway functional) where the highest level of multimerization was shown, and most stable in a recF strain where they show the lowest level of multimerization.

Most of the naturally occurring ColE1-like plasmids were found to show low level of multimerization even in recBC sbcA cells, indicating that those naturally occurring plasmids must encode a function which reduces the level of multimerization. Similarly, the maintenance of ClODF13 requires, in addition to the replication region of the plasmid, the presence of two other regions designated par A and par B. It was found that par A contributes weakly to plasmid stability, par B on the other hand, eliminates multimeric plasmid forms and results in the stable maintenance of the plasmid at least in copy number mutant plasmids.

1.3.4 Site-Specific Recombination

Site-specific recombination is a recombination event involving two defined sites that occurs independently of generalized recombination. While there is a requirement for some sequence homology between the participating duplexes for site-specific recombination, the region of homology is much smaller than that required for generalized recombination. A specific site-specific

recombination enzyme, usually encoded by a gene adjacent to the recombination site acts to mediate site-specific recombination (Nash & Pollock 1983). All characterised site-specific recombination systems act by a simple breakage-reunion reaction.

Three types of recombination events can be sponsored by site-specific recombination depending on the relative positions of both participating site: intermolecular fusion, intramolecular inversion and intramolecular deletion (resolution) . In fact, for systems so far analysed there are representatives for each type of event. Moreover, the choice of recombination event is crucial to the biological function of each system, and despite similarities between these recombination systems, they have evolved diverse biological roles. Because the energy states of the reaction substrates and product ought to be very similar, the presence of a functional site-specific recombination system should generate an equilibrium mixture of similar quantities of reactants and products. Though this appears often to be the case for the inversion in the (flip-flop) recombination systems, the other systems have evolved ways of shifting this equilibrium.

Bacteriophage lambda integration and excision is the archetype of site-specific recombination involving conservative breakage and reunion of DNA strands. Integration of lambda *in vivo* requires lambda *Int* protein and the products of two host encoded genes, *him*A and *him*D, which is in combination form integration host factor (IHF). It normally occurs between *att*P, a region of about 240 bp on lambda, and *att*B a region of some 25 bp on the bacterial chromosome, generating two hybrid sites *att*L and *att*R. Though *att*P and *att*B are not

identical, they share a 15 bp homologous 'core' sequence within which recombination occurs. Binding of Int and IHF occurs on both arms of attP surrounding the core, whereas Int and IHF bind solely to core of attB. The reverse recombination reaction, between attL and attR, which leads to lambda excision in vivo, requires Int protein, IHF and a second protein specified by the xis gene.

Active partitioning system in the low copy number P1 plasmid which is maintained at one copy per cell, will not ensure the stable inheritance of the plasmid if the plasmid undergoes generalized recombination and form dimers, because a dimer molecule can not be partitioned to two cells and that will result in the appearance of plasmid-free cells at a very high rate, but it was found that fewer than one of 10^4 cell divisions of E. coli cells that harbour P1 produce a plasmid-free cell (Rosner, 1972). P1 has been shown to encode a site-specific recombination system consisting of a site called lox P and adjacent gene called Cre, whose trans-acting product catalyzes recombination between lox P sites, (Stenberg and Hamilton, 1981). Deletion of the fragment containing lox P site and Cre gene resulted in a sharp decrease in the stability of P1.

Conversion of P1 dimers to monomers is reversible but it would appear that intramolecular resolution between two lox P sites is more efficient than the intermolecular fusion reaction.

Plasmid RP4 in E. coli is found predominantly as monomeric DNA molecules. In this state it is stably inherited. Deletion derivatives were isolated which show high level of instability and which form multimers by recA-dependent recombination. Two distinct mechanisms were found to be involved in the reduction of the level of

multimerization: one involves the plasmid-encoded primase. The second mechanism acts by efficiently resolving any plasmid multimers to monomers via a site. Inactivation of primase or deletion of the site leads to low levels of plasmid instability (0.2 or 0.9% per generation, respectively); elimination of the resolution site and primase causes a high rate of plasmid loss (5-7% per generation) and the accumulation of plasmid multimers (Grinter, 1985). It was proposed that multimerization disrupts normal partitioning and the results of only limited numbers of RP4 molecules being actively segregated at cell division are consistent with such a proposal.

Some invertable DNA regions have different functions and genetic organization, but the actual inversion systems are closely related in both the recombination sites and the amino acid sequence of the recombinases. Pin, Gin, Hin and Cin are more than 60% homologous at the amino acid level (Plasterk et al, 1983). To take just one example the Hin protein can invert a 1 kb region of the S. typhimurium chromosome by intramolecular recombination across a 14 bp inverted repeat to determine which of the two flagellar types is synthesized (Simon et al, 1980). Inversion for these systems occur relatively rarely; there would be little sense in rapid switching from one host range or flagellar type to another. This in turn appears to be a function of low-level expression of the recombinase genes (Plasterk et al, 1983).

Transposons of the Tn3 family specify site-specific recombination systems that act on directly repeated copies of the recombination site res (Arthur and Sherratt, 1979). Such molecules are generated in the first step of intermolecular transposition and are rapidly resolved

to the normal transposition end products by the site-specific recombination. The related transposons Tn1, Tn3 and Tn1000 share interchangable resolvase and *res* sites. A sub-group of class II elements including Tn501, Tn1721 and Tn21, share interchangable site-specific recombinations functions between themselves but not with Tn3 (Diver *et al*, 1983).

There is one catagorized example of an eukaryotic site-specific recombination system. Most strains of *Saccharomyces cerevisiae* harbour several copies of a plasmid termed the 2 micron circle DNA. This plasmid contains two 599 bp precise inverted repeats across which a site-specific inversion occurs *in vivo*, promoted by the plasmid encoded function called FLP (Broach, 1982). A functional recombination site is contained within a 63 bp region which contains three binding sites for FLP, arranged as two direct repeats and one inverted repeat about an 8 bp 'core' (Vetter *et al*, 1983). There is in fact remarkable homology between FLP binding sites and *Cre* binding sites of P1; in fact FLP is reported to bind at *lox*P but not recombine two of these sites. Thus, there may have been strong conservation of this recombination system during evolution from prokaryote to yeast.

The fact that ColE1 and CloDF13 encode determinants for monomerization of multimers suggested that they too might encode their own site-specific recombination functions, and that these might be directly implicated in stability. This belief was strengthened by the observation that the Tn3 *res*/resolvase system is capable of stabilizing ColE1 and CloDF13 derivatives lacking their own stability determinants (Summers and Sherratt, 1984; Hakkaart *et al*, 1984).

Analysis of ColE1 unstable mutants defective in monomerization led Summers and Sherratt to define a region *cer*, that was necessary for monomerization.

The fact that *cer* worked independently of the host generalized (*recBC*, *recE*, *recF* and *recA* pathways) recombination-system strongly suggested that *cer* was in fact a site-specific recombination function.

The work in this thesis defines a region in plasmid ColK *ckr* that is required for stability and appears to act in a similar way to *cer* in plasmid ColE1. The results provide more insight into the nature and mechanism of action of *cer* and *ckr*.

CHAPTER 2

MATERIALS AND METHODS

2.1 Strains

All bacterial strains listed are derivatives of *E. coli* K-12.

Name	Genotype	Source
AB1157	thr ara leu proA tsx lac his strA argE thi	Howard-Flanders *et al* 1964
JC8679	As AB1157 but recB21 recC22 sbcA23	
ΔM15	Δ [lac pro] thi ø 80 lacZ M15	U. Ruther

2.2 **All plasmids other than those whose construction is described in this thesis are listed in table 2.1**

TABLE 2.1

Name	Plasmids Description	Phenotype	Size (kb)	Source or Reference
ColK	naturally occurring	Cka^+ Ika^+ Mob^+	7.2	D J Sherratt.
pACYC184	Vectors derived from P15A	Cm^rTc^r	4.0	Chang and Cohen 1978.
pBR322	Vector derived from pMB8	Ap^rTc^r	4.36	Bolivar *et al*, 1977.
pAT153	pBR322 *Hae*II deletion	Ap^rTc^r	3.7	Twigg and Sherratt, 1980.
pUC8 and pUC9	Vectors derived from pBR322	Ap^r	2.7	Vieira and Messing, 1982.

2.3 Chemicals and Enzymes

Chemical	Source
General chemicals and organic compounds	BDH, Hopkins and Williams, Koch-Light Laboratories, May and Baker.
Media	Difco, Oxoid.
Biochemicals	Sigma.
Antibiotics	Sigma.
Agarose	Sigma, BRL.
Restriction enzymes and DNA modifying enzymes	BRL.
X-Gal (5-Bromo-4chloro-3indolyl- D-galactoside)	BRL.
Dimethyl Sulphide	Aldrich Chemical Co. Ltd.
Hydrazine	Kodak.
SDS	Serva.

2.4 Basic Media

L-broth: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 g glucose; made up to 1 litre with distilled water, adjusted to pH7.0 with NaOH.

Nutrient agar: 25 g Oxoid No. 2 nutrient broth, 12.5 g agar; made up to 1 litre in distilled water.

L-agar: As L-broth but without glucose and solidified by adding 10 g/l of oxoid No. 1 agar prior to autoclaving.

Iso-sensitest agar: 23.4 g isosensitest broth, 12.5 g agar; made up to 1 litre in distilled water.

Supplements: Where required growth supplements were added at the following concentrations: glucose 2 mg/ml, amino acids at 40 µg/ml, thymine at 20 µg/ml, thyamine (B1) at 2 µg/ml, casamino acids 5 mg/ml, plus 40 µg/ml tryptophan when required.

2.5 Sterilization

All growth media were sterilzed by autoclaving at 120°C for 15 min; supplements, gelatin solution and buffer solutions at 108°C for 10 min, and $CaCl_2$ at 114°C for 10 min.

2.6 Buffers

E: 0.04 M Tris, 0.02 M NaAc, 0.001 M EDTA, pH8.2 with acetic acid; usually kept as 10 X concentrate.

TBE(X10): 108 g Tris, 55 g Boric acid, 9.3 g EDTA; made up to 1 litre with distilled water. The pH should be about 8.3.

TE: 10 mM Tris-HCl, 1 mM EDTA, pH7.5.

FSB: 10% Ficoll (w/v), 0.5% SDS (w/v), 0.06% bromophenol blue (w/v), 0.06% orange G (w/v), made up with buffer E.

SCFSB: 2.5% Ficoll (w/v), 1.25% SDS (w/v), 0.015% bromophenol blue (w/v), 0.015% orange G (w/v); made up with buffer E.

Lytic mix: 2% Triton X-100, 0.05 M Tris pH8.0, 0.06 M EDTA pH8.0; made up with distilled water.

Low salt restriction buffer-LSRB(X10): 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DTT, pH7.4. Used for restrictions with HaeII, HaeIII, HpaII.

Medium salt restriction buffer-MSRB(X10): 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DTT, 50 mM NaCl, pH7.4. Used for restrictions with AccI, AluI, BamHI, ClaI, HincII, HindIII, PstI, PvuII, Sau3A.

High salt restriction buffer-HSRB(X10): 500 mM Tris-HCl, 100 mM $MgCl_2$, 1 M NaCl, pH7.4. Used for restriction with EcoRI, SalI.

TaqI restriction buffer(X10): 100 mM Tris-HCl, 60 mM $MgCl_2$, 60 mM 2-mercaptoethanol, 1 M NaCl.

Core buffer-supplied by BRL(X10): 500 mM Tris-HCl, 100 mM $MgCl_2$, 500 mM NaCl. Used for restrictions with HaeII, HaeIII, HindIII, PstI.

Ligation buffer(X10): 0.67 M Tris-HCl pH7.6, 0.1 M $MgCl_2$, 10.0 mM EDTA ph9.0, 0.1 M DTT. Made up to a final volume of 192 µl by addition of 132 µl 1M Tris-HCl pH7.6, 20 µl 1 M $MgCl_2$, 20 µl 100 mM EDTA pH9.0, and 20 µl 1 M DTT.

Buffered ATP(X10): 0.67 M Tris-HCL pH7.6, 4 mM ATP.

Phage buffer: 7 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 0.25 g $MgSO_4.7H_2O$, 15 mg $CaCl_2.2H_2O$, 1 ml 1% gelatin solution made up to 1 litre in dH_2O.

Davis-Mingioli salts (X4): 28 g K_2HPO_4, 8 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 1 g Na_3 citrate, 0.4 g $MgSO_4$. $7H_2O$; made up to 1 litre with distilled water.

D/M minimal medium: 100 ml of D/M salts, 4 ml of 20% glucose, 20 ml of CAS amino acid 20%, 2 ml of tryptophane 4 ug/ml and 2 ml of thimine (B1) 1 ug/ml were added to 300 ml of water agar.

2.7 Growth Conditions

Liquid cultures were grown in L-broth at 37°C with aeration unless otherwise stated. 'Stationary phase cultures' were small liquid cultures, usually 2 ml, grown without shaking at 37°C overnight.

Plates contained 25 ml agar with the appropriate supplements and were incubated at 37°C for 16-18 hr unless otherwise stated. All dilutions prior to plating were made in phage buffer.

Bacterial strains were stored on Nutrient agar slopes at room temperature or in 50% L-broth, 40% glycerol at -20°C. Inocula from the slopes or glycerol cultures were streaked out onto selective plates of the appropriate types and after growth at 37°C the plates were stored at 4°C for up to 1 month before fresh streaks were made. These plates provided a source of inocula for the 2.5 ml stationary phase cultures.

2.8 Antibiotic Selections

Throughout this work the standard antibiotic concentrations given below were used in both liquid and plate selections, unless otherwise stated.

Antibiotic	Resistance Encoded by	Selective conc.	Stock soln.
Ampicillin (Ap)	transposon	50 μg/ml	5 mg/ml in water
Streptomycin (Str)	chromosome	50 μg/ml	5 mg/ml in water
Tetracycline (Tc)	Plasmid	10 μg/ml	1 mg/ml in 0.1 M NaOH-made fresh

The appropriate volume of antibiotic stock solution was mixed into the medium, agar solutions having been pre-cooled to 50°C. The medium employed was dictated by the required selections as follows:

Ap, Tc, Str - these selections were on Nutrient agar, L-agar or in L-broth.

Indicators: X-Gal (5-Bromo-4chloro-3indolyl-βD-galactoside) was used at 20 μg/ml in L-agar. A stock solution in methanol was made up freshly.

This indicator was used in conjunction with cloning DNA fragments into the poly-linker of pUC8. Ligated DNA was used to transform ΔM15 and transformants were plated onto Ap, X-Gal plates.

2.9 Transformation With Plasmid DNA

A fresh overnight culture of the recipient strain in L-broth was diluted 1:40 in 20 ml of L-broth. The culture was grown at 37°C, with shaking, for about 90 min, or until there were about 2 x 10^8 cells/ml. The time required to reach this stage is strain dependent, *rec*A^+

The time required to teach this stage is strain dependent, recA$^+$ strains grow much faster than recA$^-$ derivatives. Cells were pelleted at 12,000 g for 5 min, resuspended in 10 ml cold 50 mM $CaCl_2$ and left in an ice/water bath for 10 min. The cells were pelleted again, resuspended in 1 ml of cold 50 mM $CaCl_2$ and left in an ice/water for between 1 and 5 hr. The competence for DNA uptake by recA$^+$ cells increases for up to 24 hr on ice at this stage (Dagart and Ehrlich 1979). 0.2 ml of the cell suspension were mixed with up to 50 µl of DNA solution (DNA was usually diluted in dH_2O - 0.1 µg DNA is sample for each transformation). The DNA/cell mixture was mixed well then incubated in an ice/water bath for 20 min; at 37 ^oC for 7 min then ice/water again for 30 min. 1.0 ml of fresh L-broth was added to each tube and incubated at 37^oC, shaking, for 30-120 min to allow expression of antibiotic resistance. All antibiotic selections required expression times of at least 90 min, except ampicillin, which needs less than 30 min for expression. 100-500 µl aliquots were plated onto selective agar and incubated overnight at 37^oC.

2.10 Tests for Colicin Production and Sensitivity

i. Colicin Production

The strain to be tested was stabbed onto a nutrient plate and grown overnight at 37^oC. The cells were then killed by exposure to chloroform vapour for 10 minutes. The plates were then overlaid with

2.5 molten soft agar at 46°C containing 0.2 ml stationary phase colicin sensitive indicator bacteria and then reincubated overnight. Colicin production was detected as a zone of killing in the indicator lawn around the stab. Mitomycin C can be added to the nutrient plates (0.05 g/ml) to induce cells to greater colicin production.

ii. **Colicin Sensitivity - Immunity Stab Test Plates**

0.2 ml stationary phase culture of the test strain was mixed with 2.5 ml soft agar and this pipetted onto an immunity test plate. The test plates were prepared by inoculating colicin K producing strain onto sections on nutrient agar plates. After overnight incubation at 37°C the inocula were killed by exposure to chloroform vapour. Colicin sensitive strains show clear zones of killing around the dead inocula. Immune or resistant strains are recognised by a reduced, turbid or absent zone.

iii. **Preparation of Crude Colicin K**

A crude colicin K extract for use in selective plates was prepared by inoculating 100 ml of L-broth with a colicin K producing strain and growing with vigorous shaking at 37°C to 2 x 10^8 cells/ml. Mitomycin C was then added to a concentration of 1 g/ml and growth continued overnight. The cells were pelleted, resuspended in 10 ml phage buffer and lysed by the addition of 2 ml chloroform and 30 seconds vortexing. 0.5 ml aliquots of this crude extract were stored at -20°C and retained activity for at least 12 months. Colicin plates

were prepared by adding the extract to molten nutrient agar at 46°C before pouring. Each colicin preparation was assayed to find the optimum dilution factor: this was usually about 1:1000.

2.11 Assay of Plasmid Stability

A single colony of the test strain was picked from a selective plate and inoculated into 2.5 ml L-Broth (containing appropriate antibiotic to select against plasmid-free cells) and incubated overnight at 37°C. This culture was diluted 10^{-6} fold into D/M minimal medium and grown into stationary phase. The cycle of dilution and growth in minimal medium was performed five times. Each cycle constituted 20 generations. From each stationary phase culture, samples were diluted and spread onto antibiotic-free agar and the resulting colonies were tested for plasmid content using differential staining by starch/iodine plate assay for ampicillin resistance plasmids (Bayko and Ganschow, 1982).

2.12 Purification of Plasmid DNA

i. Cleared Lysates

Cleared lysates were prepared from 100 ml cultures. Cells were harvested by centrifugation at 12,000 g, 4°C for 10 min. The cell pellet was resuspended in 3.3 ml cold 25% sucrose/0.25M Tris-HCl, pH8.0. 0.67 ml of fresh lysozyme solution, made up at 10 mg/ml in 0.25 M Tris-HCl, pH8.0, were added and the cell suspension swirled frequently, on ice, for 10 min. 1.3 ml of 0.25 M EDTA, pH8.0 were added and swirled again, while on ice, for 5 min. 5.3 ml of lytic mixed were added gently to the cell suspension, the lysate was swirled gently to ensure thorough mixing. The cells were judged to have lysed when the mixture was clear and viscous; this usually took about 5 min. The lysate was centrifuged at 43,000 g, 4°C for 20 min to pellet the chromosomal DNA and membranous material. The supernatant, containing mainly plasmid DNA, was carefully decanted - this is referred to as the cleared lysate. The cleared lysate was purified further by CsCl/EtBr equilibrium centrifugation.

ii. CsCl/EtBr Equilibrium Centrifugation

For DNA of greater purity, cleared lysates were run through CsCl/EtBr gradients. For each tube 5.0 g CsCl, 4.83 ml cleared lysate, 0.33 ml EtBr(3 mg/ml), 0.1 ml 0.2 M Na_2HPO_4 were mixed and

placed in Beckman quickseal tubes. The density of the mixture was checked (it should be about 1.58/cm^3), the tubes were filled with liquid paraffin and heat sealed. They were centrifuged in a Ti50 or Ti70 rotor in a Beckman L-8 ultracentrifuge at 48 Kcpm for 16 hr at 15°C. Two bands were formed within the gradient, the lower, denser one contains covalently closed DNA. This band was removed by inserting a syringe needle through the side of the tube and slowly drawing off the band laterally; this is enabled by previously piercing the top of the tube with a hot needle. EtBr was removed from the DNA by repeated extraction with a butan-1-ol. The solution was diluted with three volumes of water, then precipitated by addition of two volumes of ethanol at -20°C for one hour. The DNA was collected by centrifugation at 32,000 g, 4°C for 25 min. The pellet was washed with 70% ethanol/H_2O, dried, then resuspended in 500 μl TE.

iii Birnboim-Doly DNA Purification

The two previous methods each had their problems. Phenol extracted DNA could not be guaranteed to restrict; 'caesium DNA' takes two days preparation and is an expensive procedure for the isolation of, for example, putative recombinant plasmids prior to their restriction. The Birnboim-Doly method was designed for rapid purification of small quantities of plasmid DNA. I scaled up the procedure and introduced an additional purification step in order to purify equivalent amounts of plasmid DNA. Plasmid DNA purified was 14 kb or less. The purified plasmid DNA is suitable for restriction, subsequent ligation, end labelling and sequencing.

The basic principle of this method is alkaline denaturation of linear DNA molecules. Cells are lysed with NaOH/SDS; chromosomal DNA is denatured, but not covalently closed DNA. On neutralisation the mass of chromosomal DNA renatures and aggregates, proteins complex with SDS; when centrifuged the supernatant contains only covalently closed DNA and low molecular weight RNA (Birnboim and Doly 1979).

25 ml overnight cultures were harvested by centrifugation at 12,000 g, 4°C for 5 min. The supernatant was discarded, and any residual supernatant withdrawn using a pasteur pipette connected to a vacuum pump. The cell pellet was resuspended in 1 ml 50 mM glucose, 25 mM Tris-HCl, pH8.0, 10 mM EDTA, 4 mg/ml lysozyme added on the day, and then incubated at 22°C for 5 min. 2 ml of a fresh solution of 0.2 M NaOH/1% SDS were added, gently mixed, and left on ice for 5 min. To neutralize, 1.5 ml precooled 5 M KAc, pH4.8 (3 M KAc pH'd with acetic acid) were added, mixed gently and left on ice for 5 min. The crude lysate was centrifuged at 12,000 g, 4°C for 5 min, then the supernatant removed (approx. 4.5 ml). An equal volume of a 1:1 phenol/chloroform mix was added and mixed. The phases were resolved by centrifugation at 5,000 g for 5 min. The upper aqueous layer was removed and to it was added 2 volumes of 95% ethanol/H_2O. This was mixed and left at room temperature for 2 min. The solution was centrifuged at 32,000 g, 4°C for 25 min. The pellet was resuspended in 8 ml 70% ethanol/H_2O and this solution centrifuged at 32,000 g, 4°C for 15 min. The pellet, containing RNA and closed circular DNA was dried and then resuspended in 200 µl TE and 5 µl RNase solution (RNase 1 mg/ml in TE). This solution was left at room temperature for 25 min.

iv. Single Colony Cleared Lysates (referred to as SCCL)

This technique provides a quick method for analyzing the total DNA content of a particular clone. The single colonies (isolated from transformation) were 'patched' onto selective plates. Clumps of cells were resuspended in 100 µl of SCFSB. These suspensions were left at 42°C for 15 min. The lysates were spun in an Eppendorf or Sarstedt microfuge for 15 min; 50 µl of supernatant was loaded directly onto agarose gels for visualization of the DNA content.

2.13. DNA Electrophoresis Through Gels

Vertical gel kits held two glass plates measuring 16 cm x 16 cm, and separated by perspex spacers of 3 mm (1 mm for acrylamide gels). The gel liquid was poured between the two plates; 3 mm teflon combs (1 mm thick perspec for acrylamide gels) with 10 or 15 teeth were pushed into the top displacing the gel material to produce sample pockets, when removed from the set gel. Electrophoresis was from top to bottom with the cathode at the top.

'Mini-gels' were poured in horizontal gel kits measuring 10 cm x 10 cm; the gel liquid was poured to a depth of 3 mm in an area 10 cm x 8 cm allowing the electrodes to be uncovered at each end. An eight tooth 1 mm comb was placed in the still molten gel and removed when set. the cathode was at the pocket end of the gel.

i. Agarose Gels

Sigma agarose (type II, medium EEO) and buffer E were used for electrophoresis unless otherwise stated. Agarose gels for SCCL, for checking DNA concentrations and for mini-gels were made up at 0.9% (w/v); for restriction gels 1/2% agarose was usually used. The agarose/buffer E mixture was heated to 100°C until completely molten, then cooled to 52°C for gel pouring. DNA samples were mixed with 5 µl FSB and loaded with a 50 µl Finn pipette. The gel was run with buffer at anode and cathode at between 25 and 100 V until the blue die reached the bottom, unless otherwise stated. The relative separation between bands increases when gels are run at low voltage for longer times, but bands tend to be more diffuse. Restriction gels were usually run at room temperature at 80 V for 5 hr; other gels were run at room temperature at either 70 V for 6 hr, or overnight at 25 V. The gels were stained in gel running buffer containing 0.5 µg/ml EtBr for 30 min. Gels were viewed and photographed using a 35 mm SLR camera (Ilford HP5 film) with a red filter.

ii. Low Melting Point Agarose Gels

These gels were used when isolating higher forms of plasmid DNA, or particular fragments from a restriction digest which were to be used in cloning.

The agarose (1%) was dissolved in E buffer at 65°C and was cooled to 37°C. The molten agarose was poured into a mini-gel kit and cooled. After the gel had set, the comb was removed and the gel was

immersed in TE buffer. The samples (10-20 µl) were loaded and the gel was run at 5 V/cm at 4°C for 2-4 hr. The gel was stained and photographed as above.

A band could be excised and melted at 65°C. This was diluted in 1 x TBE and used for transformation or ligation.

iii. Polyacrylamide Gels

Optimal resolution of small DNA fragments was achieved by analyzing restriction digests of plasmid DNA on polyacrylamide gels. 10% (w/v) acrylamide, made up in TBE, was used to analyze small fragments of 10-300 bp; 5% (w/v) acrylamide was used to assay for larger fragments, 80-500 bp; and 3.5% (w/v) used for fragment sizes between 100-1000 bp. Acrylamide solutions (kept as 20% acrylamide, 1% bisacrylamide stock) were polymerized by the addition of 0.36 ml 10% (v/v) TEMED and 0.72 ml 10% (w/v) APS in 60 ml total volume. Gels were run with TBE buffer at anode and cathode at 30 mA at room temperature until the yellow dye reached the bottom of the gel. The gel was removed from the running apparatus, separated from the glass plates, then stained and photographed as previously described.

iv. Interpretation of Gel Data

The distance migrated is related to the size of a molecule of a given conformation; small molecules run the furthest. The distance migrated by a given DNA molecule is dependent on its conformation; supercoils generally run fastest and open circles slowest, with linear

molecules between the two. Above a certain size, all linears run at the same rate, therefore large plasmid linears run faster than corresponding supercoils. Large linear fragements of chromosomal DNA appear as a single band.

The size of linear restriction fragments was estimated from graphs of the $\log_{10}$ molecular size plotted against the distance travelled, according to the formula:

$$\log M = C \times 1/D \qquad \text{(Helling } \underline{\text{et}} \text{ } \underline{\text{al}}\text{, 1974)}$$

M = molecular size in bp, D = distance migrated, C = a constant

Molecular weight standards were obtained by restriction of λcI857Sam7 DNA, or pBR322 DNA (Philippsen et al, 1978, Haggerty and Schleif 1976, Sutcliffe 1978).

2.14. DNA Manipulations in vitro

i. Restriction of Plasmid DNA

Restriction of plasmid DNA was performed in 1.5 ml Eppendorf tubes. The final reaction volume contained:

0.5 - 1.0 µg plasmid DNA

0.1 vol 10x appropriate restriction buffer

1 - 10 units enzyme

gelatin/water (at 0.1 mg/ml) to make up volume

Complete digestion was usually achieved in 3 hr at 37°C (65°C for Taq1). Digests were analyzed by electrophoresis through agarose or acrylamide gels.

ii. **Ligation of Restriction Fragments**

Endonucleases used to digest plasmid DNA were destroyed by heating to 65°C for 5 min. Ligations were prepared by addition of 5-15 µl of each restricted DNA, plus 5 µl ligation buffer, plus 5 µl buffered ATP, plus 1 µl ligase (prepared by E. Nimmo, this laboratory) for 'sticky ends', or 2-4 µl for blunt ends, and finally made up to 50µl with dH_2O. Final concentrations were:

67 mM Tris-HCl, pH7.6

10 mM $MgCl_2$

10 mM DTT

1 mM EDTA

0.4 mM ATP

0.1 unit ligase/µg DNA

Ligation mixtures were incubated at 14°C for 16-18 hr. These were then diluted in dH_2O for use in transformation.

CHAPTER 3

DEMONSTRATION OF A STABILITY FUNCTION IN PLASMID ColK

INTRODUCTION

ColK and ColE1 are related, naturally-occurring multicopy plasmids which are similar in size, and are compatible with each other (Warren and Sherratt 1977 and 1978). Both plasmids produce colicins which act in a similar way (Tacon *et al*, 1981), and each encodes an immunity gene which protects the cell from the colicin it produces.

There is a great similarity in the organization of colicin and immunity functions on ColK and ColE1 (fig. 3.1) as well as *mob* (plasmid mobility), *bom* (basis of plasmid mobility), *rom* (repressor of plasmid maintenance) and the origin of replication functions.

ColE1 has been found to encode a stability determinant, *cer*, which reduces multimerization of ColE1 both in recombination proficient and deficient host cells, and which can stabilize unstable cloning vectors by acting in *cis*. The *cer* function has been localized to a fragment of 0.28 kb (Sherratt *et al*, 1984), located between the colicin and *mob* genes in ColE1.

The similarities between ColE1 and ColK suggested that ColK might encode a stability function analogous to *cer* in ColE1.

The data presented in this chapter strongly suggest the existence of a stability determinant in ColK (designated *ckr*), located between the colicin and the mobility genes in ColK. This confers stability by reducing the level of multimerization, when it acts in *cis*.

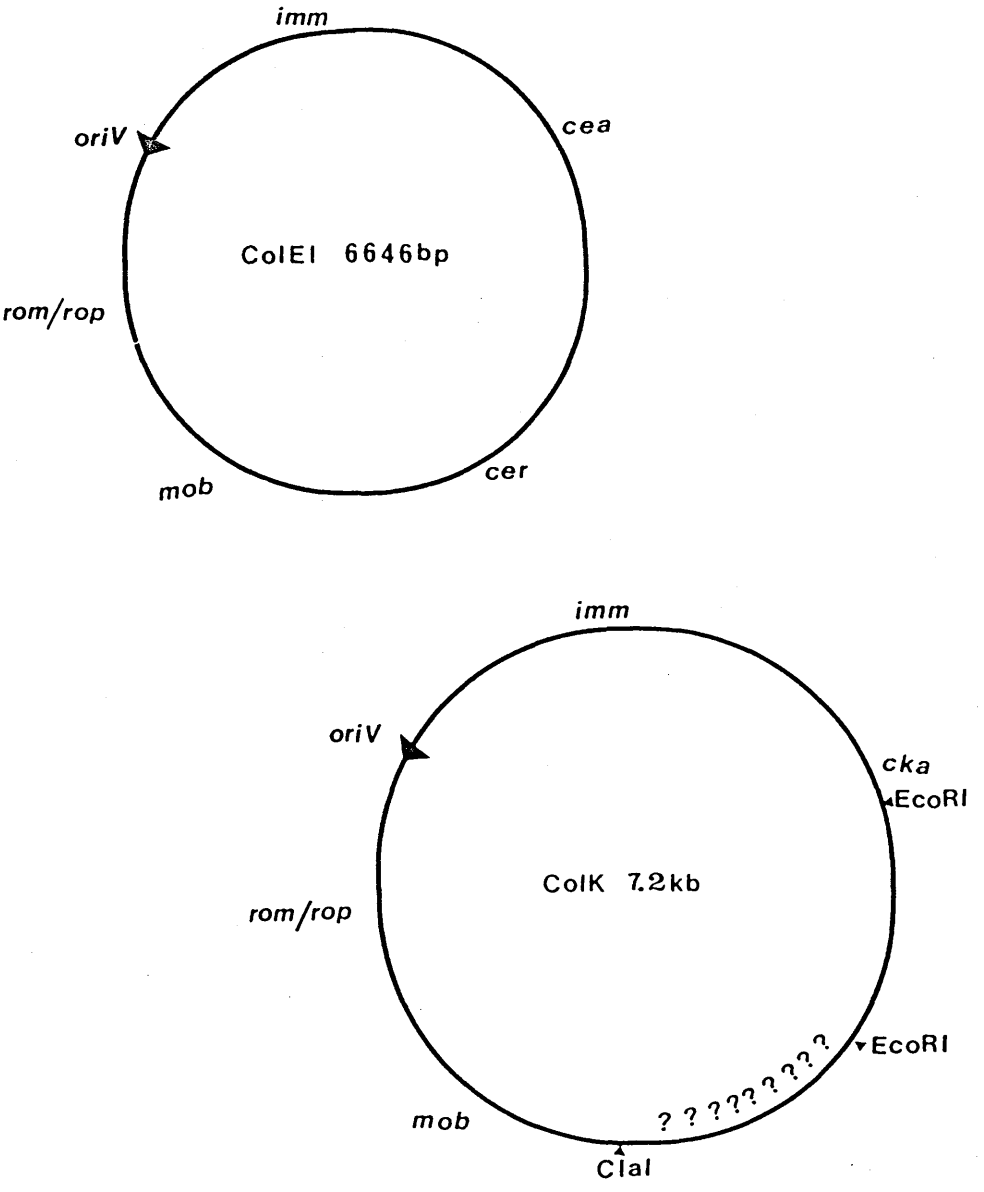


Fig 3.1 Comparison between the organisation of functions on ColK and ColE1 plasmids

3.1 Construction of ckr-Containing Plasmids

A preliminary restriction map of ColK was available when I started my project (Fig. 3.2) (Tacon et al, 1981). The region between the two EcoRI sites (about 1.5 kb) was deleted, but this deletion did not result in the loss of the stability function. This implied that any stability functions must be elsewhere in ColK.

Subsequently, a 2.1 kb EcoRI-ClaI fragment (Fig. 3.2) from ColK was cloned in pAT153. The constructed plasmid (pSY100), PAT153 and ColK were each digested with EcoRI and ClaI and analysed on a 0.85% agarose gel. A single band was observed for pAT153, which has single sites for each of EcoRI and ClaI close to each other. The second band was so small that it ran off the gel, and was not seen. Three bands were seen in the track containing digested ColK, which has two EcoRI sites and one ClaI site. Two bands were seen for pSY100. They corresponded to the large band in pAT153 and to the 2.1 kb EcoRI-ClaI fragment of ColK (Fig. 3.3). The predicted structure of pSY100 was therefore confirmed.

Later a preliminary DNA sequence of parts of ColK was determined (J. Archer, pers. comm.). A strong homology was found between ColE1 cer and part of the DNA sequence in ColK which lies within the 2.1 kb EcoRI-ClaI fragment (manuscript in preparation) (see chapter four).

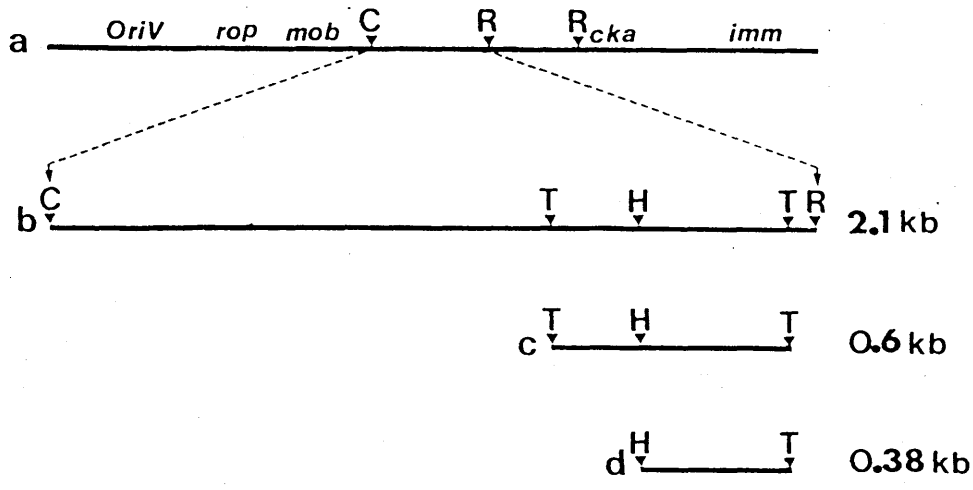


Fig 3.2 Restriction maps of ColK and Ckr$^+$ fragments

R = EcoRI

C = ClaI

Ha = HaeIII

T = TaqI

a - ColK

b - The 2.1 kb ckr$^+$ EcoRI - ClaI fragment in pSY100

c - The 0.6 kb ckr$^+$ TaqI fragment in pST10

d - The 0.38 kb ckr$^+$ TaqI - HaeIII fragment in pRY10

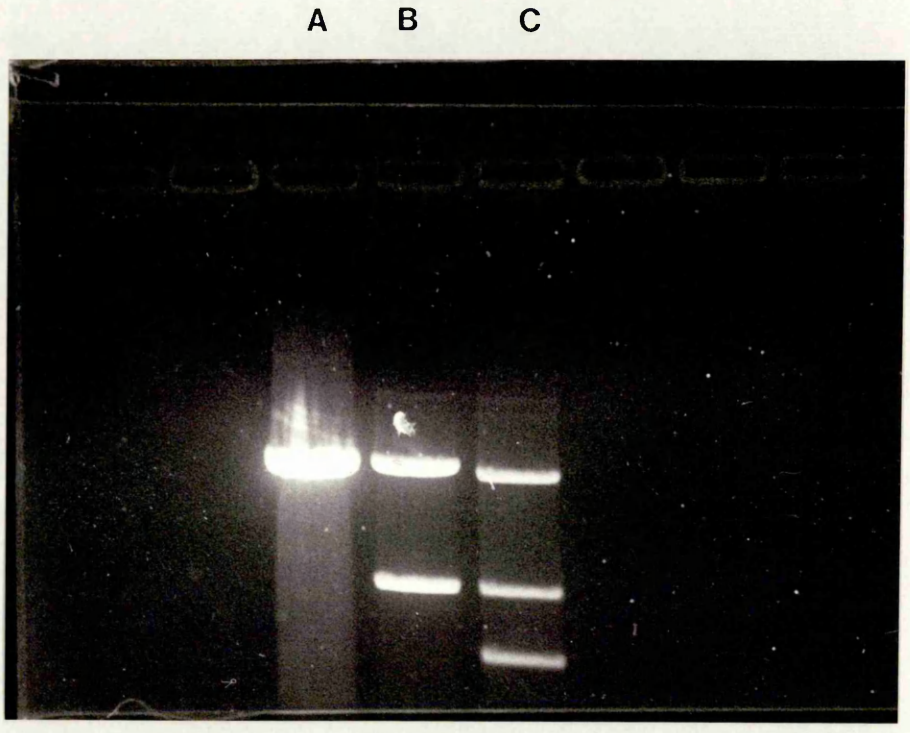


Fig 3.3 Restriction analysis of pSY100 and related plasmids

Plasmid DNAs cut with EcoRI and ClaI, run on a 0.85% agarose gel.

lane A : pAT153

lane B : pSY100

lane C : ColK

The DNA sequence provided some information about the sites of some restriction enzymes. There is a TaqI site near one of the ends of the region which is highly homologous with cer. Unfortunately the sequenced fragment was not large enough to locate another TaqI site. pSY100 was cut with EcoRI and ClaI, the 2.1 kb fragment was recovered from low melting point agarose gel, it was cut again with TaqI and ligated with pUC8 cut with Acc1. The ligated DNA was transformed into a recBC sbcA (highly recombinogenic) strain (Fishel et al, 1981). Three size classes of recombinant plasmids were seen when some transformants were analysed on gel, but only one size class showed a low level of multimerization. This new plasmid was called pST10. pST10 was cut with TaqI and compared on a polyacrylamide gel with pUC8, pSY100 and ColK which had been digested with TaqI. Four bands were seen for pST10; three of which were present in pUC8. The fourth band corresponded to a fragment of about 0.6 kb which was present in both pSY100 and ColK (Fig. 3.4).

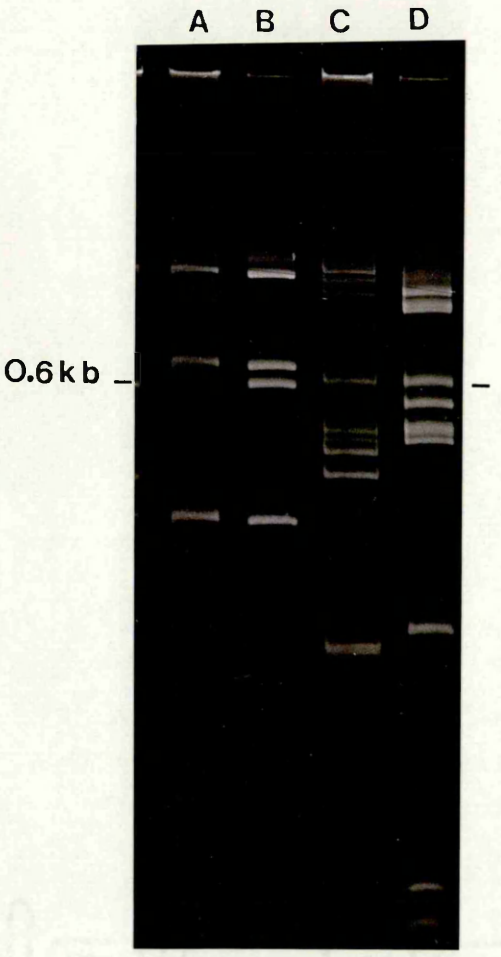


Fig 3.4 Restriction analysis of pST10 and related plasmids

Plasmid DNAs cut with TaqI and run on a 5% (w/v) polyacrylamide gel.

lane A : pUC8 lane B : pST10

lane C : pSY100 lane D : ColK

To reduce the size of the fragment containing ckr further, a new strategy was followed: pST10 was digested with an enzyme which had a unique site in the polylinker of pUC8 and a second enzyme which cuts within the insert. As a first attempt, pST10 was cut with HindIII and Sau3A, but no fragment containing the function was cloned. In the second attempt HindIII and HaeII were used but it also failed to provide a plasmid containing ckr. Finally, pST10 was cut with HindIII and HaeIII and ligated with pUC8 DNA cut with HindIII and HincII. A fragment of about 0.38 kb was cloned into pUC8. The new plasmid was called pRY10. Because the ligation of the blunt ends of HaeIII and HincII sites created a new hybrid sequence at the junction between them, which is not recognised either by HaeIII or HincII, it was difficult to confirm the identity of the fragment inserted. This was checked indirectly by restriction analysis with Sau3A. The region of ckr which is highly homologous with ColE1 cer has three sites for Sau3A. pSY100, pST10, pRY10, pKS450 (pUC9, with 377 bp cer containing fragment), pUC8 and ColK were cut with Sau3A, and analysed on a 12% polyacrylamide gel. pST10 and pRY10 showed two additional bands of 155 bp and 54 bp compared to those in pUC8. The two bands are also present in pSY100 and ColK (Fig. 3.5).

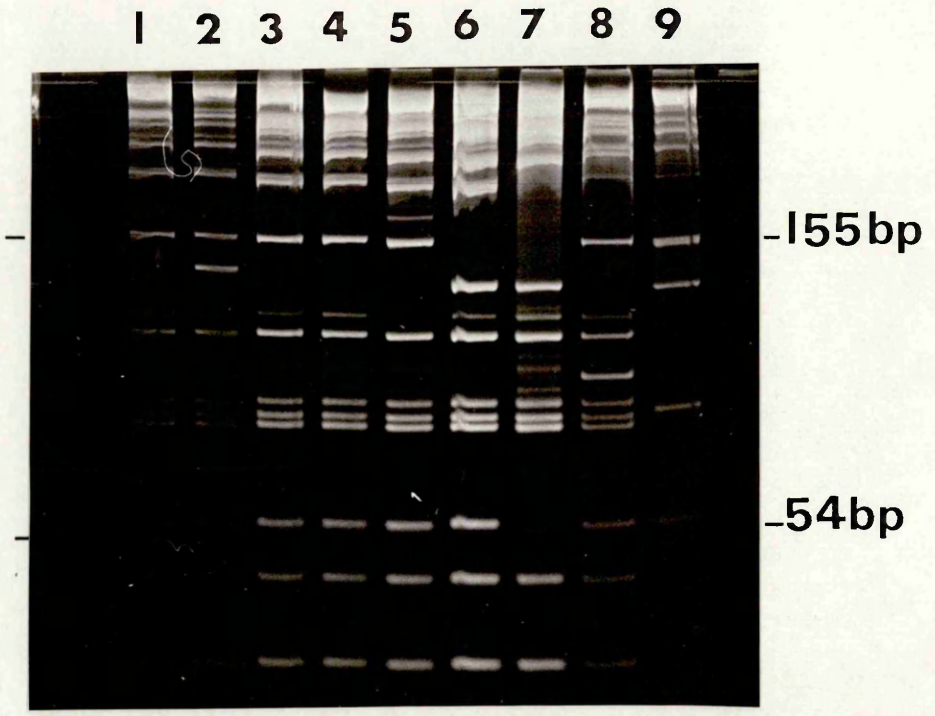


Fig 3.5 Restriction analysis of the structure of the ckr$^+$ plasmids

Sau3A was used to confirm the presence of ckr region in pSY100, pST10 and pRY10. Digests run on a 12% acrylamide gel.

lanes 1 and 2 : pRY10

lanes 3, 4 and 5 : pST10

lane 6 : pKS450

lane 7 : pUC8

lane 8 : pSY100

lane 9 : ColK

3.2 ColK Stability Function ckr Can Reduce Multimerization and Stabilize Unstable Cloning Vectors When It Acts in cis

To check the extent of multimerization of the constructed plasmids, pSY100 (pAT153, with 2.1 kb fragment from ColK), ColK and pAT153 were transformed into DS903 (recF) and JC8679 (recBC sbcA) strains which are non-recombinogenic and highly recombinogenic for plasmids respectively. In the recF strain all three plasmids showed a low level of multimerization, but in the recBC sbcA strain, pAT153 showed a high level of multimerization while ColK and pSY100 showed lower levels of multimerization (Fig. 3.6).

The extent of multimerization of pST10 (pUC8, with 0.6 kb TaqI fragment from ColK) and pRY10 (pUC8, with 0.38 kb TaqI-HaeIII fragment from ColK), was compared with that of their parent plasmid, pUC8. In the recF background all three plasmids showed a very low level of multimerization, whereas in the recBC sbcA background both pST10 and pRY10 showed no extensive multimerization, while pUC8 showed a very high level of multimerization (Fig. 3.7).

The reduction in the level of multimerization in recBC sbcA background could have been due to the prevention of the formation of multimers or to the breaking down of higher forms to monomers after they had been formed. Trimers of pAT153, pSY100, pST10 and pRY10 and dimers of pUC8 were transformed into strain DS903 (recF). The trimers

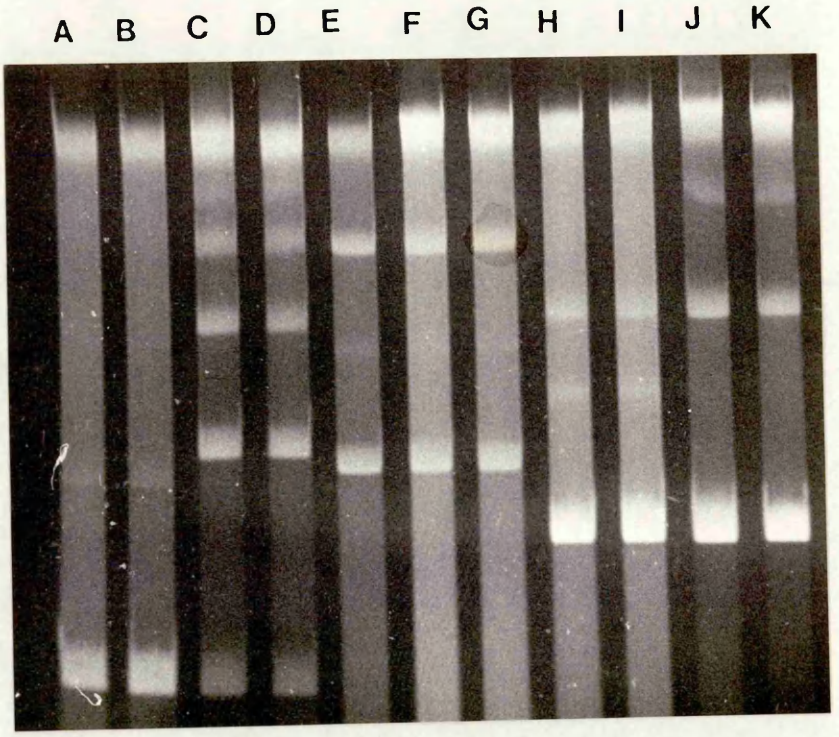


Fig 3.6 Multimerization of pSY100 and related plasmids in recombinogenic and non-recombinogenic strains

DNAs from single colonies run on a 0.85% (w.v) agarose gel

lanes A and B : pAT153 in DS903

lanes C and D : pAT153 in JC8679

lane E : ColK in DS903

lanes F and G : colK in JC8679

lanes H and I : pSY100 in DS903

lanes J and K : pSY100 in JC8679

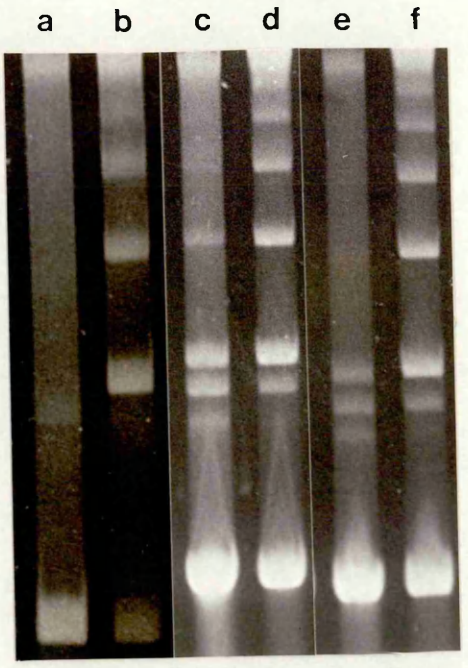


Fig 3.7 Multimerization of pUC8 and ckr$^+$ derivative plasmids

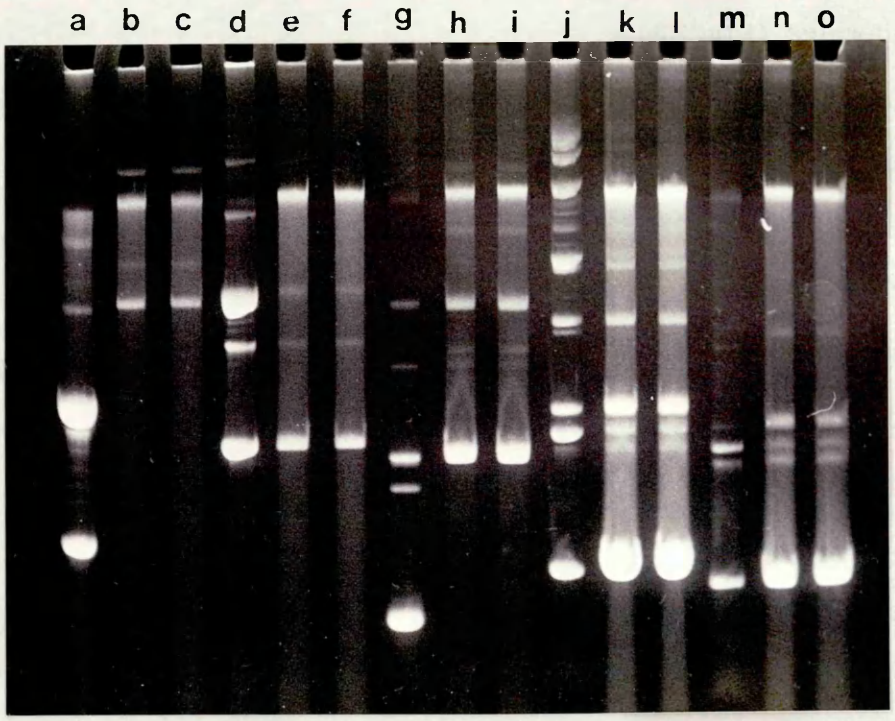
DNAs from single colonies of pUC8, pST10 and pRY10 run on a 0.85% (w.v) agarose gel.

lane a : pUC8 in DS903
lane b : pUC8 in JC8679
lane c : pST10 in DS903
lane d : pST10 in JC8679
lane e : pRY10 in DS903
lane f : pRY10 in JC8679

of pAT153 remained as trimers and the dimers of pUC8 remained as dimers, whereas trimers of pSY100, pST10 and pRY10 broke down to monomers (Fig 3.8).

Finally, plasmids containing sequences cloned from ColK were checked for stability of inheritance in the absence of drug selection, in the recBC sbcA background. Initially, pAT153 was compared with pSY100. For pAT153, plasmid-free cells appeared after 20 generations and the ratio of plasmid-free to plasmid-containing cells increased to about 85% after 100 generations. On the other hand I failed to detect any loss of pSY100 until after 80 generations when only 2% of the cells examined were plasmid-free. Even after 100 generations the ratio of plasmid-free to plasmid-containing cells did not exceed 4% (Fig. 3.9).

A similar test was done for the two pUC8 derivatives, pST10 and pRY10 . pUC8 was used as a control and, as in the case pAT153, plasmid-free cells were detected after 20 generations. Thereafter, there was a drastic increase in the proportion of plasmid-free to plasmid-containing cells reaching about 95% after 100 generations. No plasmid-free cells were detected for either pST10 or pRY10 even after 100 generations (Fig. 3.10).


a b c d e f g h i j k l m n o

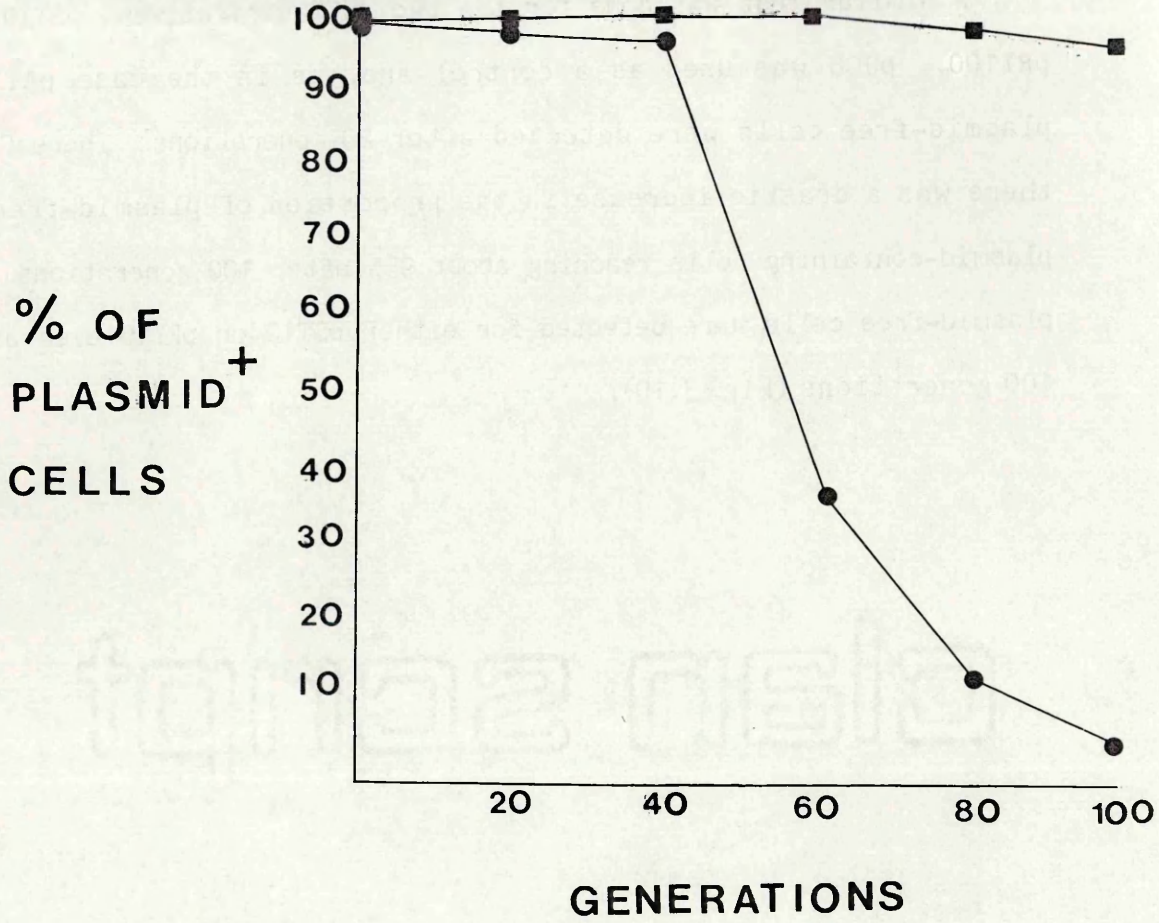

100
90
80
70
60
50
40
30
20
10
% OF PLASMID+ CELLS
20
40
60
80
100
GENERATIONS

Fig 3.8 Investigation of multimer break down of ckr$^+$ plasmids and their parent plasmids

DS903 was transformed with purified multimers of ckr$^+$ and ckr$^-$ plasmids. The multimeric state of the plasmids in the transformants was analysed on a 0.85% (w.v) agarose gel.

lane a : pAT153 monomeric marker

lanes b and c : DS903 transformed with trimers of pAT153

lane d : pSY100 monomeric marker

lanes e and f : DS903 transformed with trimers of pSY100

lane g : pUC8 monomeric marker

lanes h and i : DS903 transformed with dimers of pUC8

lane j : pST10 monomeric marker

lanes k and l : DS903 transformed with trimers of pST10

lane m : pRY10 monomeric marker

lanes n and o : DS903 transformed with trimers of pRY10

Fig 3.9 Stability of pAT153 and its ckr$^+$ derivative

(●) stability of pAT153 in a recBC sbcA host

(■) stability of pSY100 in recBC sbcA host

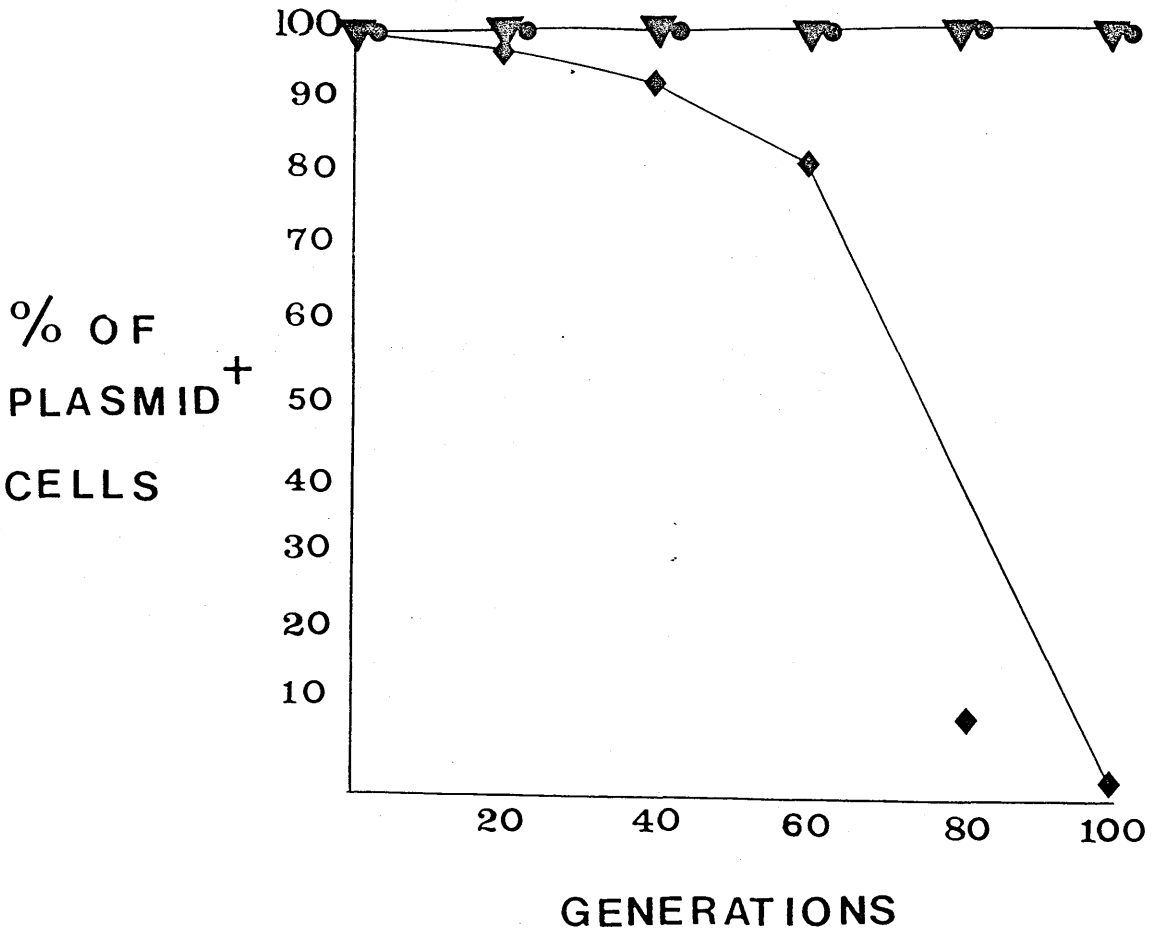


Fig 3.10 Stability of pUC8 and its ckr$^+$ derivatives

The plasmids stability was checked in recBC sbcA cell under non-selective conditions.

(◆) stability of pUC8

(▼) stability of pST10

(●) stability of pRY10

DISCUSSION

If plasmids are distributed randomly at cell division, the probability (Po) that a daughter cell does not receive a plasmid is given by the binomial distribution $Po = 2^{(1 - x)}$ where x is the number of plasmid copies per dividing cell (Novick et al, 1975).

This means that cells must have a copy number greater than 18 at division in order to lose the plasmid at a frequency of $< 10^{-5}$ per cell per generation. Since most of the cloning vectors exist at a copy number higher than 18 they should be lost at frequencies less than 10^{-5} per cell per generation. This means that, in a normal stability test no plasmid-free cells should be detected for any of the multicopy vectors even after 100 generations. But on the contrary most of them are very unstable and plasmid-free cells appear as early as 20 generations even in case of pUC9 which has a copy number of about 160 per cell (C. Jones, pers. comm.). Therefore, there must be factors other than the randomness of distribution of single plasmids which account for their loss at high frequencies.

It has been proposed that for plasmids which lack active partition systems (even though they have high copy number), the multicopy pool of plasmids behaves as a single unit at cell division (Tucker et al 1984). Because of that, they are lost at higher frequencies than those predicted from the random distribution model.

My data on the stability of pAT153 and pUC8 suggest that there may be competition between plasmid-free cells and plasmid containing cells when both are grown under non-selective conditions (Figs. 3.9 and 3.10). Plasmid-free cells grow faster than plasmid-containing cells

because the latter need to replicate the plasmids they contain which results in slowing down their growth rate. This competition contributes to the sharp increase in the proportion of plasmid-free cells to plasmid-containing cell during the course of stability tests for multicopy cloning vectors.

Multimerization and stability have been found to be related inversely (Summers and Sherratt, 1984) and that has been postulated to be a result of copy number variance caused by multimerization. This variance in copy number can create a subpopulation of cells containing low copy numbers of multimeric plasmids. These cells will produce plasmid-free cells.

An equal chance of initiating the replication at any plasmid origin within a cell will give replication of multimers an advantage over replication of monomers. Furthermore, monomers have been found much more stable than multimers in recF background (C. Jones, pers. comm.).

My data show that ColK encodes a stability function, ckr, which can convert higher forms to monomers. This suggests that ckr encodes at least part of a resolution system which works by recF independent recombination which occurs when two copies or more of ckr are found on the same molecule. However my data do not show whether recombination occurs at a specific site or at different sites, and do not give any evidence on whether ColK encodes its own recombination enzyme(s). Because of the sequence homology between ColE1 cer and ckr [illegible] it is likely that ckr and cer are similar in size, and since cer has been

defined to a fragment of about 0.28 kb which is too small to encode a protein of the size of resolvase in Tn3 or *Cre* in phage P1, it is unlikely that *ckr* encodes its own recombination protein. Such an enzyme might be encoded by the *E. coli* chromosome.

CHAPTER 4

RECOMBINATION BETWEEN DIRECTLY REPEATED *cer* AND *ckr*

INTRODUCTION

The results in chapter 3 suggested that the breakdown of *ckr*$^+$ plasmid multimers to monomers was due to a recombination event (independent of *recA*, *recF*, *recE* and *recBC* pathways) across two identical sites within directly repeated *ckr* regions. A similar recombination event had been suggested for the breakdown of *cer*$^+$ plasmid multimers (Summers and Sherratt, 1984). This implies that recombination at *cer* and *ckr* sites could be analogous to that which occurs at sites in some other recombination systems, for example the recombination between two *lox*P sites in prophage P1.

DNA sequences of *cer* and *ckr* regions are highly homologous but not identical. It was predicted that recombination between *ckr* and *cer* is likely and could result in creating a hybrid region containing DNA sequences from *cer* and *ckr*.

The integration of bacteriophage lambda into the chromosome of *E. coli* normally occurs between *att*P (a region on lambda of about 240 bp) and *att*B (a region of some 25 bp on the bacterial chromosome), generating two hybrid sites *att*L and *att*R (Weisberg and Landly, 1983). Similarly, occasional intermolecular recombination which results in the integration of P1 into the *E. coli* chromosome occurs between *lox*P on P1 and *lox*B on the *E. coli* chromosome, *lox*P consists of a 13 bp inverted repeat surrounding an 8 bp 'core' region (containing the crossover site), *lox*B is a 10 bp inverted repeat, sharing 8 bp homology with *lox*P (Austin *et al*, 1981; Hoess *et al*, 1982).

The cointegrate resolution system of Tn3 and Tn1000 are closely related; the recombinases of the two transposons are interchangeable (Reed 1981). A hybrid resolution site has been constructed *in vivo* by resolvase-mediated recombination between Tn3 and Tn1000 *res* sites. By comparison of the sequence of this hybrid site with the sequences of the parental sites, it was possible to localize the crossover to a region of 19 bp.

4.1 Intramolecular Recombination Between Two Directly Repeated Copies of *cer* Results in Deletion

A 377 bp *cer*$^+$ *Hpa*II fragment from ColE1, (Summers and Sherratt, 1984), was inserted separately in two different orientations into the *Nar*I site (a unique restriction site outside the polylinker) in pUC9 to construct pKS450 and pKS451 (Fig 4.1). The same fragment had already been cloned between *Eco*RI and *Hind*III sites in the polylinker of pUC9, to construct pKS410. Plasmid pKS410 was cut with *Eco*RI and *Hind*III, and DNA from the fragment containing *cer* was ligated separately with pKS450 and pKS451 (both cut with *Eco*RI and *Hind*III). This resulted in cloning the *cer*$^+$ *Eco*RI-*Hind*III fragment into pKS450 and pKS451. The new recombinant plasmid pSY450 resulting from cloning the fragment into pKS450 was about 3.4 kb in size (Fig. 4.2 and 5.1), which is simply the sum of the pKS450 (3 kb) plus the *cer*$^+$ fragment (about 0.4 kb). The other recombinant plasmid (pSY451) resulted from cloning the *cer*$^+$ fragment into pKS451 was also about 3.4 kb in size, but in *recF* cells transformed with pSY451, a 2.9 kb plasmid, designated pSY452 was recovered (Fig. 4.2).

In pSY450, the two copies of *cer* were in the opposite orientation, therefore as expected, no deletion occurred when the

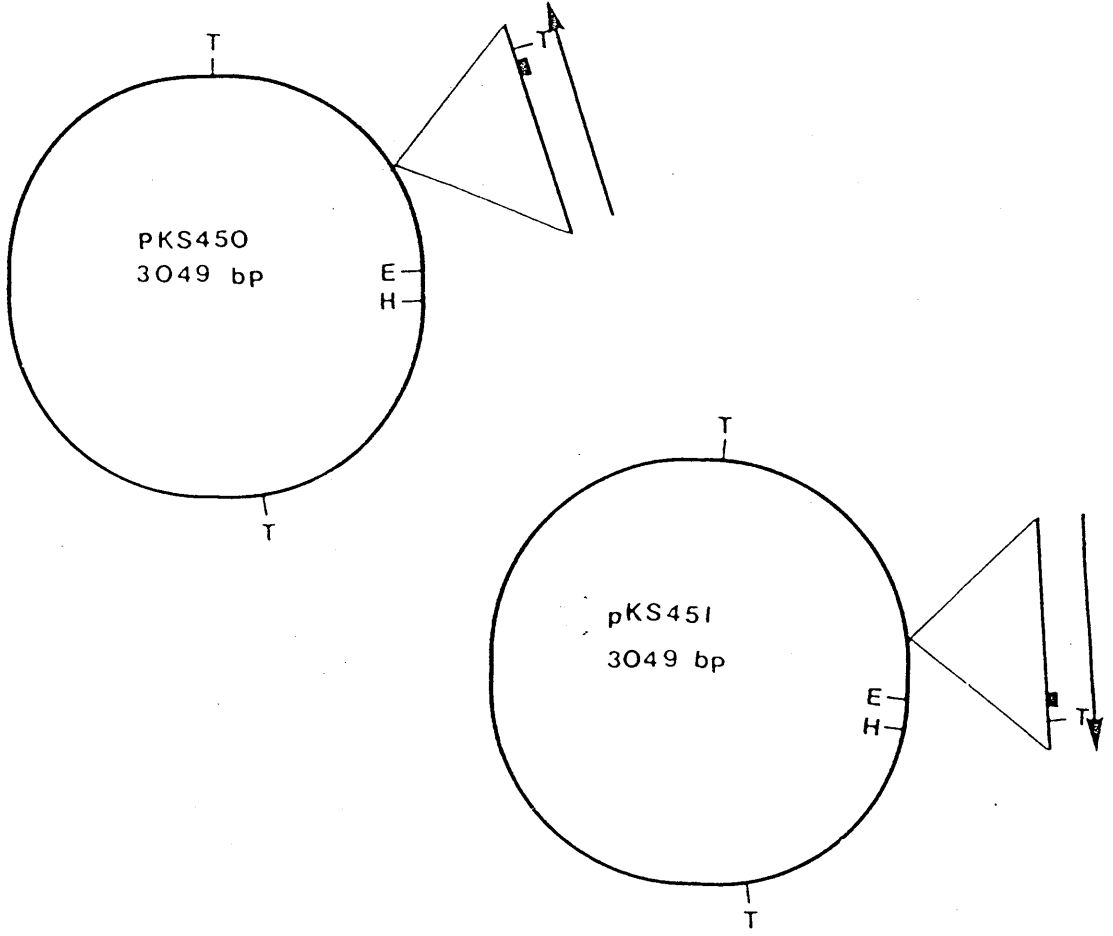


Fig 4.1 Structure of pKS450 and pKS451

T = TaqI

E = EcoRI

H = HindIII

plasmid was transformed into recF cells. In pSY451 the two copies of cer are in the same orientation and a fragment of about 0.5 kb was lost as a result of recombination between the two copies of cer.

The restriction analysis of pSY452 (isolated from recF cells), showed that the plasmid had lost all of the restriction sites in the 161 bp which separated the two copies of cer in pSY451 plus the restriction site of one of the two copies of cer which were present in pSY451. This implied that pSY452 had lost a copy of cer and the fragment separating the two cer sites.

The stability of pSY452 was compared with those of pUC9 and pKS451 (all in recBC sbcA host cells), and the result of the test showed that pSY452 was as stable as pKS451 while pUC9 was very unstable (Fig. 4.3). In JC8679 (recBC sbcA cells) the level of multimerization of pSY452 was much lower than that of pUC9, indicating that the copy of cer in pSY452 was functional.

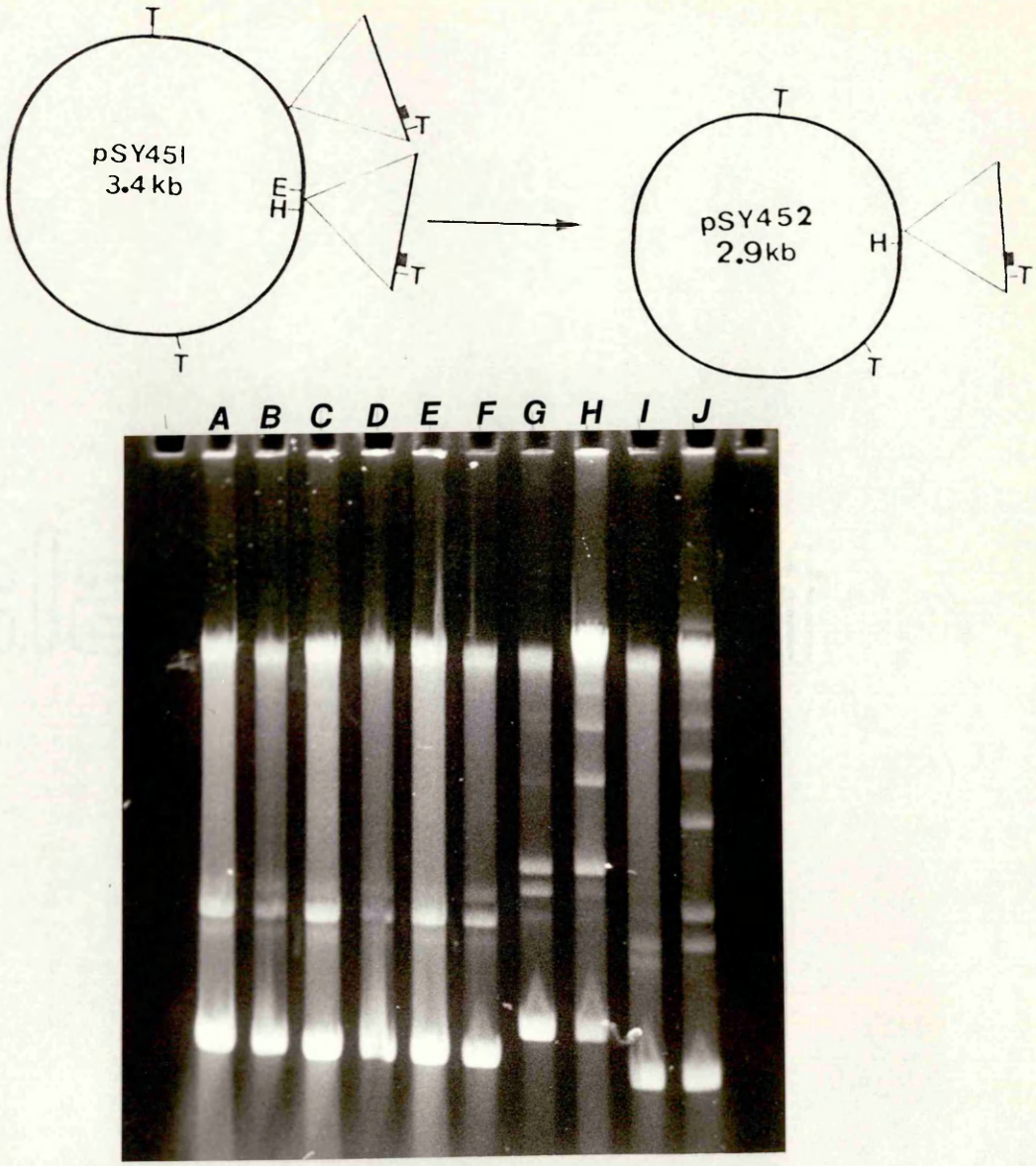


Fig 4.2 Construction of 2 cer plasmids and derivation of pSY452 by in vivo deletion

DNAs of single colonies of DS903 cells transformed with plasmid DNA, run on a 0.85% (w.v) agarose gel.

lanes A and B : pKS450 lanes C and D : pKS451

lanes E and F : pKS410 lanes G and H : pSY450

lanes I and J : pSY452 lanes H and J in JC8679

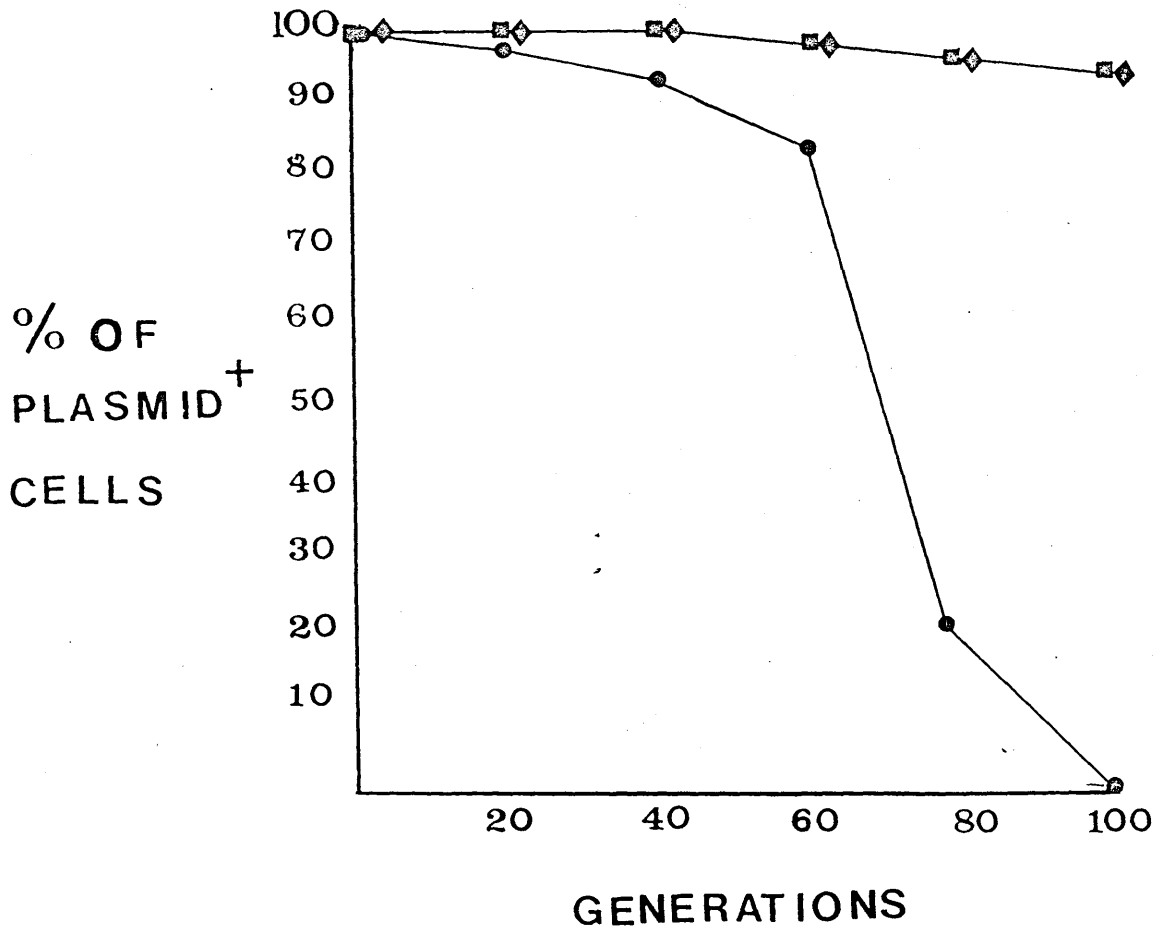


Fig 4.3 Stability of *cer*$^+$ plasmids and their parent plasmid

Plasmids were tested for stability in *recBC sbcA* background and under non-selective conditions.

(■) stability of pKS451

(◆) stability of pSY452

(●) stability of pUC9

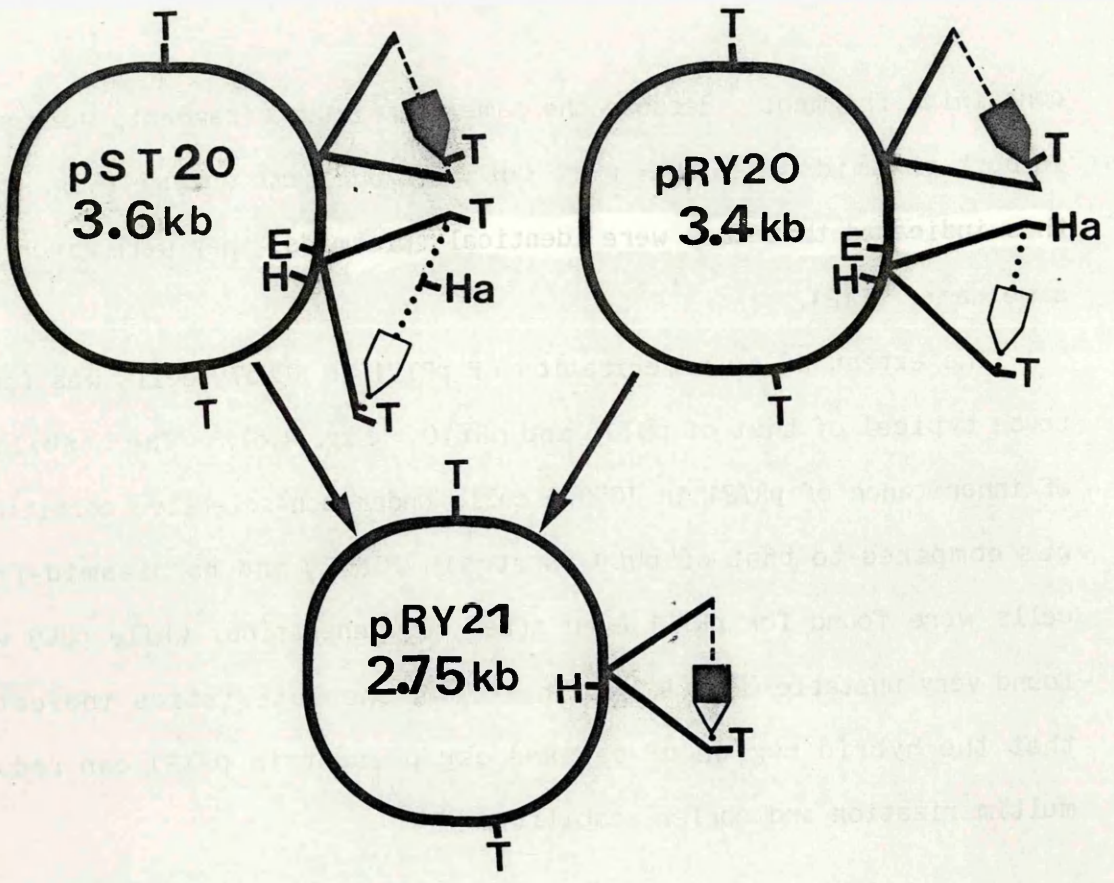
4.2 Intramolecular Recombination Between cer and ckr

To investigate recombination between cer and ckr, two plasmids were constructed each of which contained a copy of cer and a copy of ckr in direct repeat. The first plasmid, pST20, (3.6 kb) (Fig. 4.4) was constructed by cloning the ckr^+ EcoRI-HindIII fragment from pST10 (pUC8, with the 0.6 kb ckr^+ TaqI fragment in the polylinker), into the polylinker of pKS451. The ckr^+ EcoRI-HindIII fragment from pRY10 (pUC8, with the 0.38 bp ckr^+ TaqI-HaeIII fragment in the polylinker) was cloned into the polylinker of pKS451 to construct the second plasmid which was called pRY20 (3.4 kb) (Fig. 4.4).

The size difference between pST20 and pRY20 was 3.6-3.4 = 0.2 kb, which is the difference between the 0.6 kb ckr^+ TaqI fragment cloned in pST20 and the 0.4 kb ckr^+ TaqI-HaeIII fragment cloned in pRY20. In pST20 the size of the fragment between any two homologous points in cer and ckr (which might be the crossover sites) was about 0.85 kb and the region between the same two points in pRY20 was about 0.65 kb When pST20 and pRY20 were introduced into DS903 cells, two plasmids of the same size (about 2.75 kb) were isolated. Both plasmids were studied by restriction analysis. The plasmid derived from pST20 was found to have been deleted in the 161 bp fragment (which separated cer and ckr fragments in pST20) plus a region of about 550 bp from the fragment containing ckr and a region of about 140 bp from the cer-containing fragment. The plasmid derived from pRY20 had been deleted in the 161 bp fragment plus a region of about 140 bp from the cer-containing fragment and a region of about 350 bp from the ckr-

containing fragment. Because the same restriction fragments were seen in both plasmids when they were cut with the same enzyme (Fig. 4.5), they were given the same name, pRY21.

The extent of multimerization of pRY21 in JC8679 cells was found to be typical of that of pST10 and pRY10 (Fig. 4.6). The stability of inheritance of pRY21 in JC8679 cells under non-selective conditions was compared to that of pUC9 in strain JC8679 and no plasmid-free cells were found for pRY21 even after 100 generation, while pUC9 was found very unstable (Fig. 4.7). These two characteristics indicated that the hybrid region of cer and ckr present in pRY21 can reduce multimerization and confer stability.


pST20
3.6kb
T
E
H
Ha
pRY20
3.4kb
pRY21
2.75kb


a b c d e f g h l j k l

Fig 4.4 Construction of ckr and cer containing plasmids and derivation of plasmids containing ckr-cer hybrid regions

T = TaqI

E = EcoRI

Ha = HaeIII

(----) ColE1 sequence

(....) ColK sequence

( ) cer sequence

( ) ckr sequence

DNAs of single colonies of DS903 and JC8679 cells transformed with plasmids, run on a 0.85% (w.v) agarose gel.

lane a : pST10 in DS903

lane b : pST10 in JC8679

lane c : pRY10 in DS903

lane d : pRY10 in JC8679

lane e : pST15 in DS903

lane f : pST15 in JC8679

lane g : pRY15 in DS903 } Not discussed
lane h : pRY15 in JC8679 }

lane i : pRY21 (derived from pST20) in DS903

lane j : pRY21 (derived from pST20) in JC8679

lane k : pRY21 (derived from pRY20) in DS903

lane l : pRY21 (derived from pRY20) in JC8679

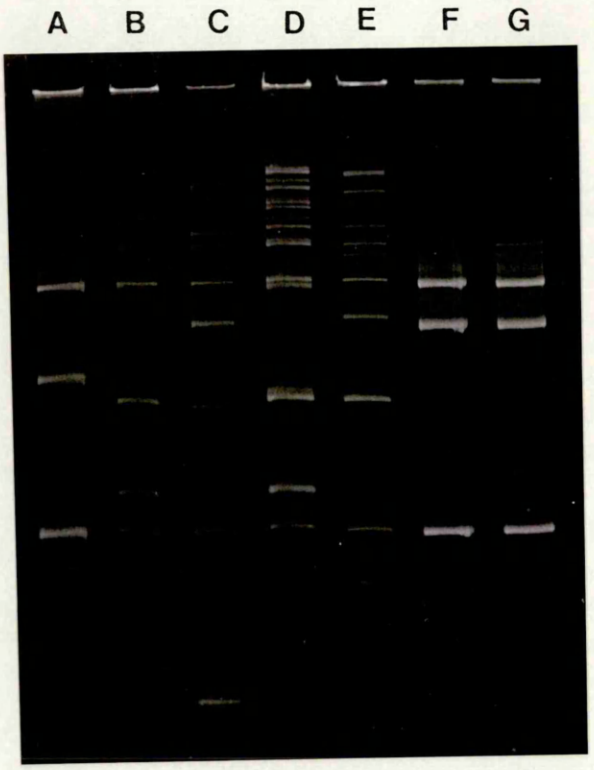


Fig 4.5 Restriction analysis of plasmids derived by *in vivo* deletion from pST20 and pRY20

Plasmid DNA was restricted with *Taq*I and run on 5% (w.v) polyacrylamide gel

lane A : pUC8 lane B : pKS450

lane C : pKS451 lane D : pST15

lane E : pRY15 lane F : pRY21 derived from pST20

lane G : pRY21 derived from pRY20

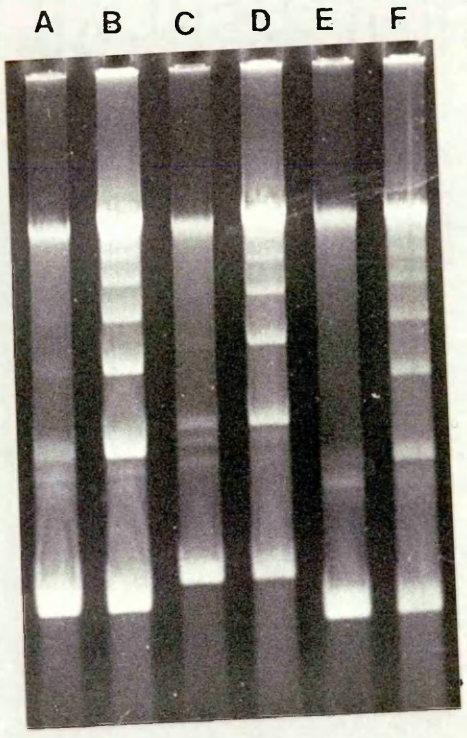


Fig 4.6 Multimerization of pRY21 and related plasmids

DNAs from single colonies of JC8679 and DS903 cells transformed with plasmid DNA run on a 0.85% (w.v) agarose gel.

lane A : pST10 in DS903

lane B : pST10 in JC8679

lane C : pRY10 in DS903

lane D : pRY10 in JC8679

lane E : pRY21 in DS903

lane F : pRY21 in JC8679

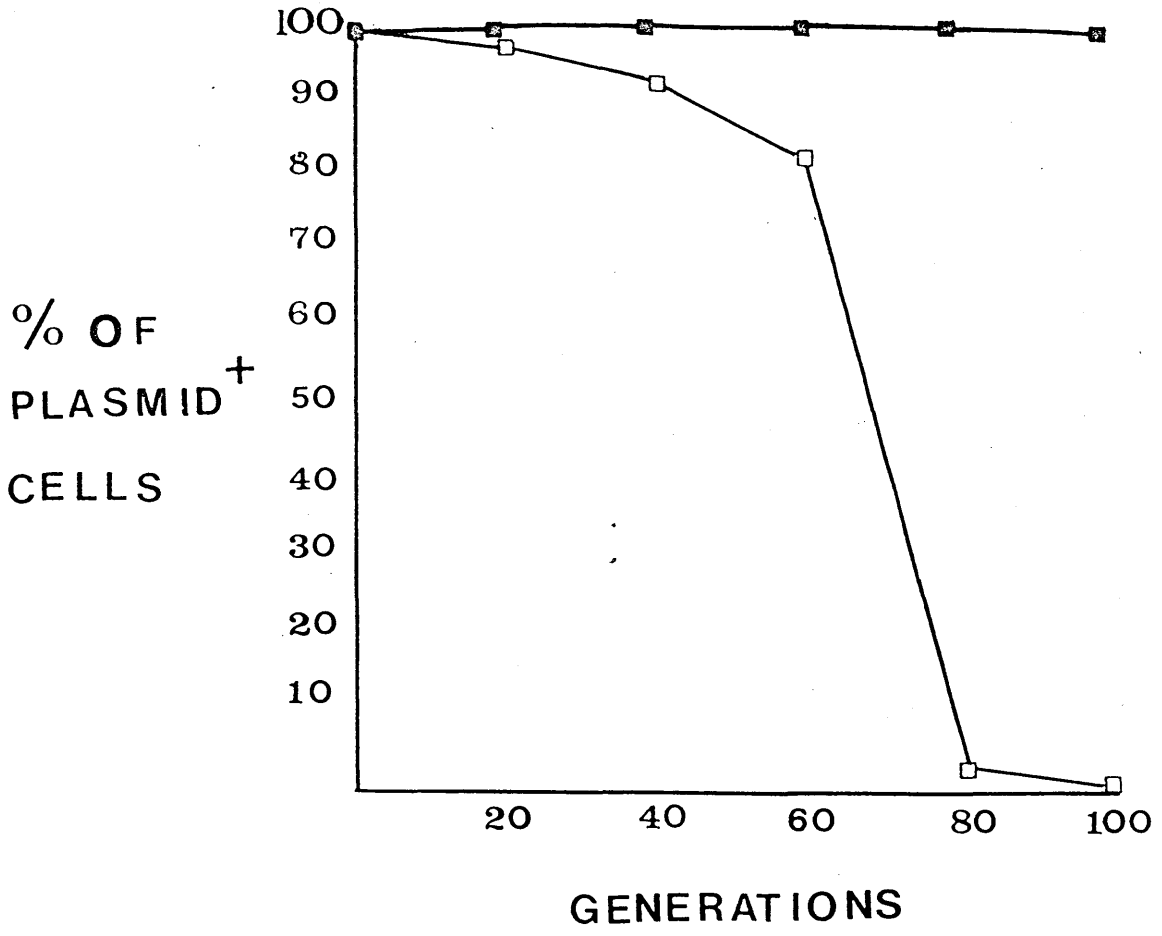


Fig 4.7 Stability of plasmid containing ckr-cer hybrid region and its parent plasmid

The stability of the plasmids was checked in recBC sbcA background and under non-selective conditions.

(□) stability of pUC9

(■) stability of pRY21

The deletion in the plasmid containing two copies of *cer* in direct repeat (pSY451), showed that pSY451 was broken down to two molecules each of which contained one copy of *cer*, one molecule was pSY452 and the second molecule was the 161 bp fragment plus one copy of the *cer* fragment. These two molecules were the products of the recombination between the two copies of *cer*. Note that only one product was recovered because it contained the origin of replication of pUC9.

This experiment can be considered as a control for the experiment investigating *cer* and *ckr* in direct repeat on the same plasmid, in which the recombination between the *cer* and *ckr* resulted in breaking down the plasmid containing a copy of *cer* and a copy of *ckr*, to two smaller molecules each of which contained a hybrid region incorporating sequences from *cer* and *ckr*.

The restriction analysis of pRY21 failed to acheive the main aim of the experiment which was the localization of the crossover site. Therefore, in this laboratory the DNA sequence of ten hybrid regions from ten independent isolates of pRY21 was determined. This showed that recombination between *cer* and *ckr* occurs within a 35 bp region of exact homology (Fig. 4.8), (Yaish *et al*, manuscript in preparation). This strongly indicates that recombination between *cer* and *ckr* and therefore, by implication, between pairs of homologous sites, is site specific.

Resolution by most of the known site-specific recombination systems requires a recombination enzyme, Tn3 and P1 encode their own recombination enzymes, resolvase and *Cre* respectively, while some stains of *E. coli* encode the product of *pin* gene which acts on the *gin*, *hin*, and *cin* inverted repeats (Plasterk *et al*, 1983).

Because *cer* and probably *ckr* DNA sequences are too small to encode a protein similar in size to *Cre* or Tn3 resolvase, it has been proposed that the *E. coli* chromosome encodes a gene (designated *xer*) which produces a product which mediates recombination at *cer* and also at *ckr*. A xer^- strain with a mutated *xer* region was constructed, and cer^+ plasmids were introduced into it. They showed an extensive level of multimerization which implied that the efficient resolution by *cer* shown by xer^+ strains was absent (manuscript in preparation).

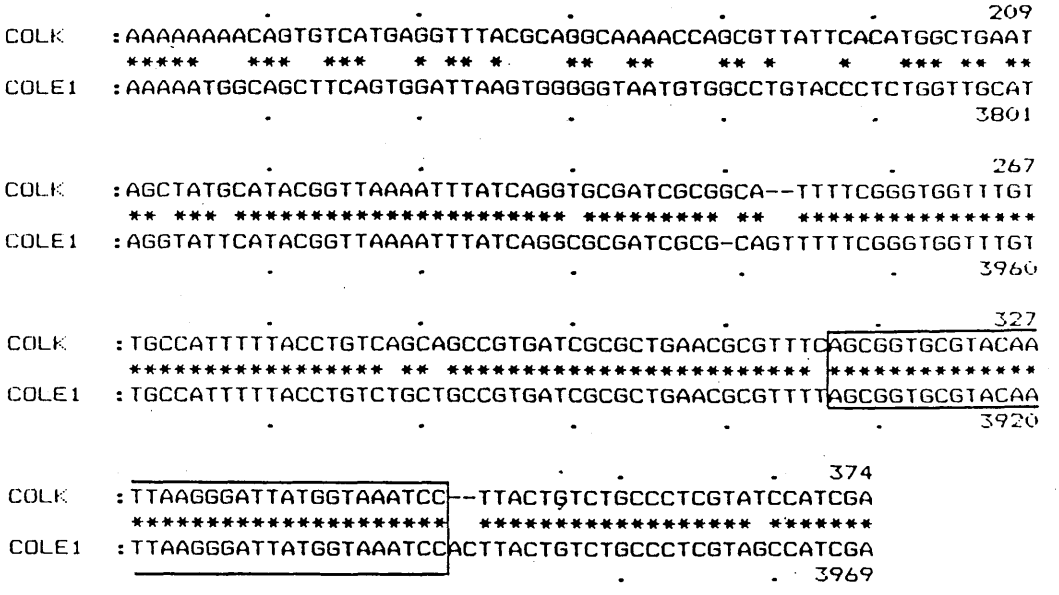


Fig 4.8 Sequence comparison of ckr and cer regions

The 'core' regions of ckr and cer have been aligned to maximize homology with gaps inserted (-). Homologous nucleotides are indicated by stars. Sequences numbering regers to ColK and ColE1 sequences irrespective of inserted gapps. The regions share about 82% homology when so aligned. The crossover site between ckr and cer lies within the boxed region of 35 bp, from 313 to 348 on ckr coordinates. (D. Summers pers. comm.)

CHAPTER 5

RECOMBINAION BETWEEN INVERTED COPIES OF *cer* AND *ckr*

INTRODUCTION

Intramolecular site-specific recombination will result in deletions or inversions depending on the relative orientation of the recombination sites. For both phage lambda excision and Tn3 resolution the normal substrate consists of direct repeats of the site. There are a number of closely related systems in prokaryotes where site-specific recombination acts on inverted sites to generate inversions of the intervening DNA. Two systems have been well studied: (1) inversion of the G-region of bacteriophage Mu (Kamp *et al* 1978; Van de Putte *et al* 1980) and the C-region of the related phage P1 (Chow and Bukhari 1976) and (2) phase variation in *Salmonella typhimurium* (Silverman and Simon 1980). The *gin* (Mu), *cin* (P1) and *hin* (*S. typhimurium*) gene products can complement each other in all combinations (Kutsukake and Iino 1980; Kamp and Kahman 1981), suggesting a close evolutionary relationship between these inversion systems. More recently an invertible region in the *E. coli* chromosome has been analyzed. A function which can complement *gin* mutation was called *pin* and catalises inversion of a 1600 bp P-region (Plasterk *et al* 1983).

Inversion has been detected, albeit at relative low frequency, between two copies of Tn3 *res* in inverted orientation, and also between two copies of *res* from different transposons, one *res* from Tn3, the second from Tn1 (Dyson 1984). Heffron *et al* (1981) observed inversion when they used a substrate containing *res* sites divorced from the main body of the transposon: one *res* from Tn3, the second from Tn1000.

Recombination in cer and ckr is site specific, and causes efficient deletions when two copies of cer, ckr or one copy of each are present in direct repeat. We therefore wished to test if inversions might occur between two copies of cer or ckr if they were present in indirect repeat, and at what frequencies such inversions would occur.

5.1 Inversion of DNA Sequences Between Two Copies of cer in Inverted Orientation

Efficient resolution was observed in pSY451 (its construction described in Section 4.1) which has two copies of cer in direct repeat. To check inversion between two copies of cer in inverted orientation, pSY450A (described in Section 4.1) which contains two copies of cer in opposite orientation, separated by 161 bp, was transformed into DS903 (recF) cells, plasmid DNA was isolated after 40 generations (the shortest time feasible to allow analysis of DNA on a gel) and restricted with EcoRI and HindIII. The presence of two new restriction fragments indicated that a proportion of pSY450 had inverted. Approximately 10% of pSY450A DNA was present in the inverted form. The strain was further subcultured and analyzed after 50, 100, 150 and 200 generation (Fig. 5.1), there was almost no change in the proportion of the inverted to non inverted DNA forms which indicated that an equilibrium between both forms was reached in less than 40 generations. The inverted form of pSY450A; termed pSY450B was constructed by ligating DNA from the two faint bands containing DNA of the restriction fragments of the inverted form (Fig. 5.1). pSY450B was introduced into DS903, plasmid DNA was isolated after 40

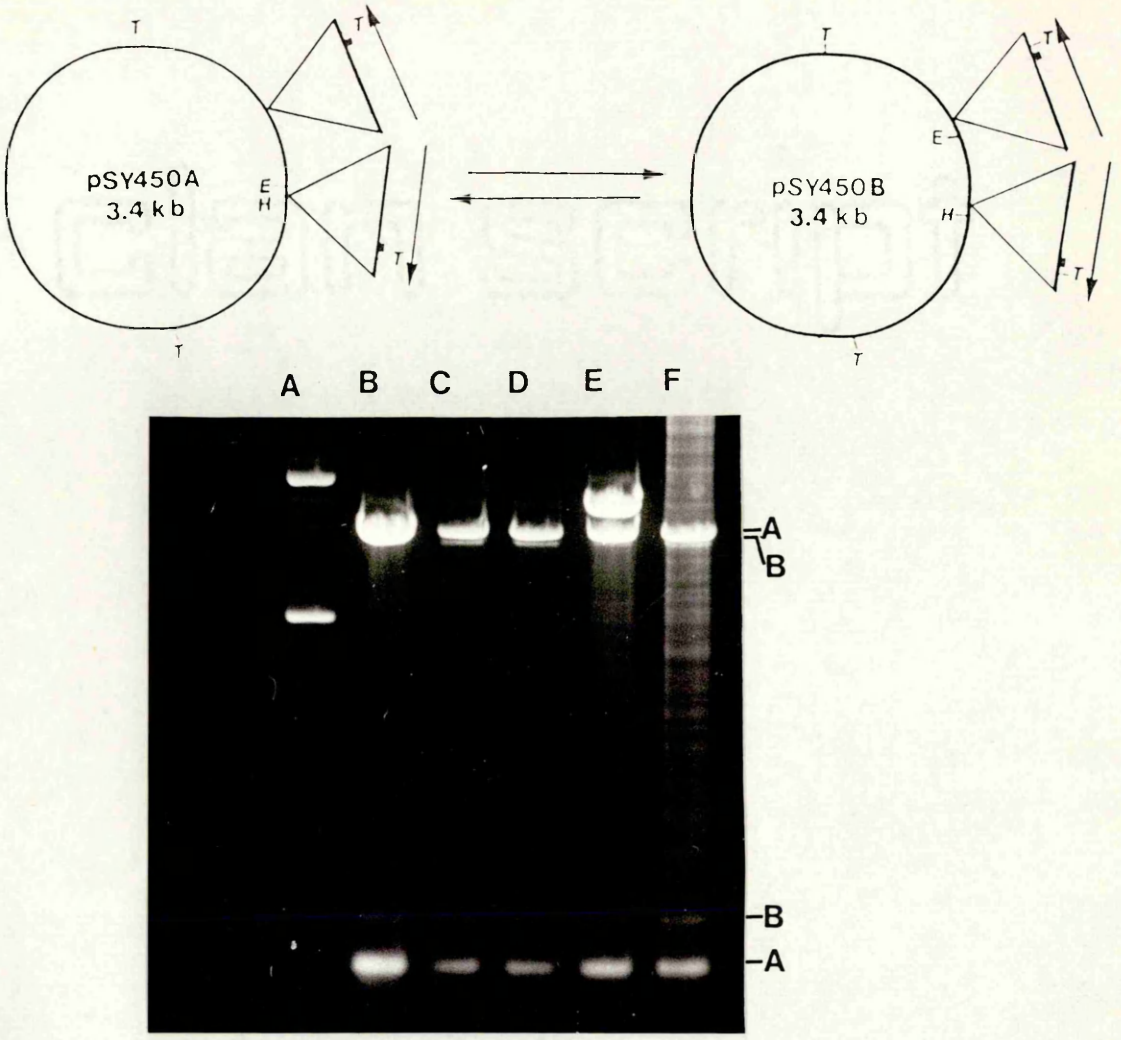


Fig 5.1 Inversion analysis of pSY450

0.85% (w.v) agarose gel

Inversion was monitored by restricting plasmid DNA with EcoRI and HindIII.

lane A pSY450 uncut

lane B after 40 generations

lane C after 100 generations

lane D after 140 generations

lane E after 180 generations

lane F after 220 generations

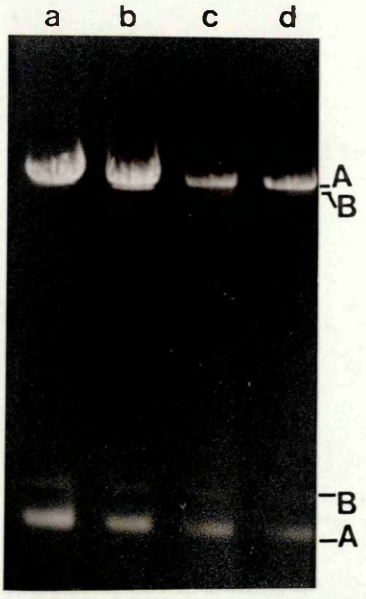


Fig 5.2 **Analysis of pSY450 inversion reversibility**

0.85% (w.v) agarose gel

Inversion was monitored by restricting plasmid DNA with EcoRI and HindIII.

lane a : pSY450 after 200 generations

lane b : pSY450B after 40 generations

lane c : pSY450B after 120 generations

lane d : pSY450B after 200 generations

generations and restricted with EcoRI and HindIII this showed that only about 10% of the DNA was present in the pSY450B form and 90% of the DNA had inverted back to pSY450A form, this proportion did not change when the strain was subcultured for 200 generations (Fig 5.2).

5.2 Inversion Results From Site-Specific Recombination Between cer and ckr

Because inversion was detected between two indirectly repeated cer copies, and since recombination between cer and ckr in direct repeats resulted in a deletion of intervening DNA, it was predicted that recombination between cer and ckr in inverted repeat might cause inversion.

A plasmid containing a copy of cer and a copy of ckr in opposite orientation was constructed by cloning the EcoRI-HindIII ckr^+ fragment from the polylinker of pST10 into the polylinker of pKS450 this plasmid was called pST15.

To check inversion, pST15 was introduced into a recF strain and DNA of pST15 was made after 40 generations and cut with EcoRI and HindIII. If there was no inversion, the cut DNA should show two bands, one of about 3 kb and the second of about 620 bp. When the cut DNA was analyzed on an agarose gel the two bands corresponding to un-inverted pSY15 were seen, but two other faint bands were also seen, one of those bands was about 400 bp and the other was about 3.15 kb. This indicated that there was some inversion in pST15 and the inverted form of the DNA was about 10%.

The strain was further subcultured, and the inverted form of the DNA was detected after 100, 150 and 200 generations but there was

almost no change in the proportion of the inverted to the non inverted DNA forms (Fig. 5.3). This indicates that the equilibrium between the two forms was reached in less than 40 generations.

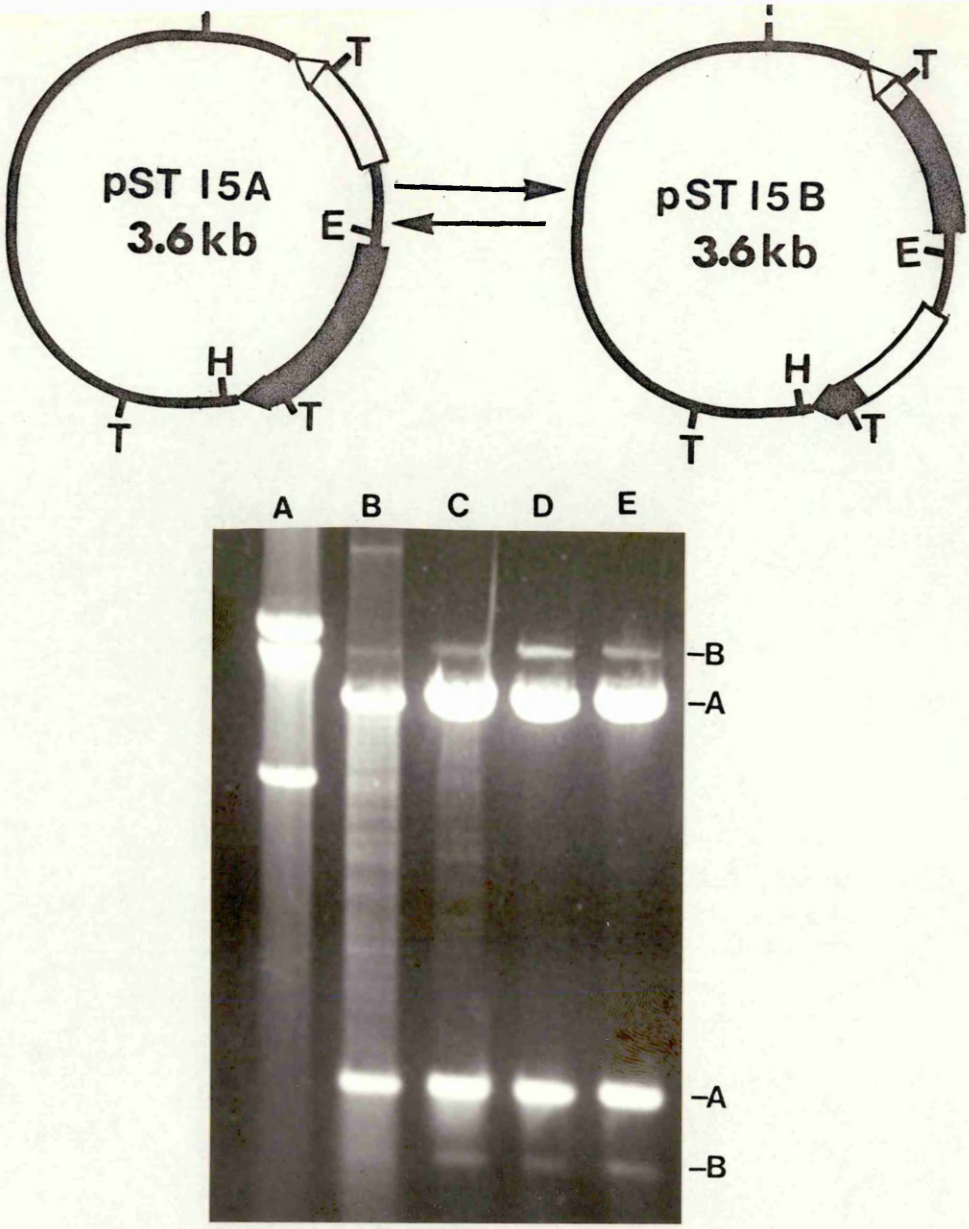


Fig 5.3 Inversion analysis of pST15

0.85% agarose gel

Inversion was monitored by restricting plasmid DNA with EcoRI and HindIII.

lane A : pST15 uncut

lane B : after 40 generations

lane C : after 100 generations

lane D : after 150 generations

lane E : after 200 generations

DISCUSSION

An equilibrium can be establised *in vivo* between two forms of an invertable substrate containing Tn1000 *res*-wt's within 50 generations (Dyson, 1984). This would imply a minimum inversion rate of 0.007 per cell per generation, approximately 20 times less efficient than the corresponding resolution reaction. And *in vitro* it is 50 times less efficient (Reed and Grindly 1981; Symington 1982).

The general conclusion is that inversion between two copies of *res*-wt is less efficient than resolution, but several factors appear to influence the former which do not noticeably affect the latter reaction. Firstly the distance between adjacent sites would appear to affect the inversion rate by an inverse proportion. Secondly inversion does not necessarily appear reciprocal *in vivo*, this means that one form of the substrate is less competitive within a cell when plasmid incompatibility effects come into play.

In contrast, it has been reported that *cin* mediated recombination between *cix* sites in direct repeat appears to be less frequent than that between inversely repeated sites (Kennedy *et al*, 1983).

Because the time needed for complete deletion between two *cer* copies and between a copy of *cer* and a copy of *ckr* in direct repeat, and also for equilibrium in their inversion reactions to be reached when they are in inverse repeat, is less than the shortest time needed to allow DNA to be analyzed on gel, it was difficult to get accurate data on the rates of both processes. Nevertheless, it gives good evidence that recombination at *cer* and *ckr* can occur irrespective of their orientation.

My results do not show any definite reason(s) for the bias in the concentrations of inverted and non inverted forms of pSY450 and pST15. It might be because inverted forms are less competitive than the non inverted forms. This might be because of inverting the fragment (which comes from pUC9) between the two sites, in which there could be a transcription termination site, and that would result in terminating transcription in the inverted plasmid.

It is not known yet what product mediates inversion in cer and ckr, and whether ColE1 and ColK encode such a product or whether it is encoded by the E. coli chromosome.

CHAPTER 6

DISCUSSION

DISCUSSION

Site-specific recombination appears to contribute to the stability of inheritance of both high copy and low copy number plasmids (Summers and Sherratt, 1984; Hakkaart *et al*, 1982 and Austin and Stenberg, 1981).

The work in this thesis demonstrates plasmid ColK is a very stable plasmid and encodes a stability determinant *ckr*, that acts by rapidly allowing the conversion of multimers to monomers by promoting site-specific recombination (independent of *recF*, *recE* and *recBC* pathways) between directly repeated sites within the *ckr* sequence.

The conversion of *ckr*$^+$ plasmid multimers to monomers, and the isolation of plasmids containing hybrid regions from *ckr* and *cer* from all cells transformed with plasmids containing a copy of *ckr* and a copy of *cer*, suggest that recombination mediated by *ckr* and between *ckr* and *cer* is very efficient. Furthermore, multimers of pRY21 (a plasmid containing *cer*-*ckr* hybrid region) are converted very efficiently to monomers, which indicates that the hybrid sequence is no less functional and efficient than either *ckr* or *cer*.

Some multimers can be seen for *ckr*$^+$, *cer*$^+$ and *cer*-*ckr* hybrid containing plasmids, this could be due to inadequate recombination efficiency to abolish all multimers or because recombination mediated by *cer* and *ckr* is reversible, if the latter is the case, intramolecular resolution would appear much more efficient than intermolecular fusion.

A typical site-specific recombination system consists of recombinase and binding site(s) for the recombinase. *ckr* seems to encode the crossover site and the binding site(s) only, the protein is suggested to be encoded by a gene carried by the chromosome and designated *xer*. A *xer*$^-$ strain in which *ckr*$^+$ and *cer*$^+$ plasmids show extensive level of multimerization has been constructed in this laboratory (G. Stewart pers. comm.).

Recombination between inversely repeated *cer* copies as well as between a copy of *cer* and a copy of *ckr* results in the inversion of the segment between the two crossover sites across which the recombination occurs. Inversion is reversible and so efficient that equilibrium between inverted form and non-inverted form of the plasmids can be reached in less than 40 generations (the shortest time to allow DNA analysis on a gel). It seems likely that inversion between *cer* and *ckr* is due to recombination which occurs at the same site as recombination between directly repeated copies of *cer* and *ckr*, which is mediated by the same gene products that mediates resolution (i.e. *xer* product).

The work in this thesis suggests some experiments which could be done in the future, like determining the minimum sequence needed for *ckr* function and this could simply be done by subcloning small fragments of the 0.37 kb *ckr*$^+$ fragments in other cloning vectors.

The construction of *ckr*$^-$ ColK deletion mutant(s) is important to confirm the direct involvement of *ckr* in the stability of ColK, and will allow a more precise determination of sequences required for recombination, it seems technically a little difficult because of the lack of useful restriction sites within the *ckr* region in particular

and in ColK in general, but one could overcome this problem by substituting a *ckr* containing fragment from ColK (e.g. the *EcoRI*-*ClaI* fragment) by a fragment (which has sticky ends to *EcoRI* and *ClaI* ends in ColK, and contains no stability function(s) from another vector.

Concerning resolution and inversion mediated by *ckr*, two plasmids could be constructed, by cloning the 0.6 kb *ckr*$^+$ *TaqI* fragment from ColK into a *TaqI* site on pST10 or pRY10 (both have *ckr*$^+$ fragments in the polylinker) by partial digest. This would result in cloning the fragment in either orientation. The plasmid containing the new *ckr*$^+$ fragment in direct repeat with the one in the polylinker could be used as a substrate for resolution. The other plasmid would have the two *ckr* copies in inverted repeat and would be a substrate for inversion.

An important experiment being conducted in this laboratory, is the construction of *xer*$^-$ mutant bacterial strain. Stability and extent of multimerization of ColK and *ckr*$^+$ recombinant plasmids in the absence of *xer* gene product could be checked by introducing them into *xer*$^-$ mutant cells.

Plasmids pSY450 (pUC9, containing two copies of *cer* in inverted repeat) and pST15, could be introduced into *xer*$^-$ cells to check whether inversion is mediated by *xer* product or not. In order to confirm that recombination between inverted repeat of *ckr* and *cer* occurs at the same crossover site at which resolution occurs, fragment(s) predicted to contain hybrid region(s) containing sequences from *cer* and *ckr*, could be sequenced, and this should define a region within which crossover occurs.

To set up an *in vitro* system for *ckr* recombination, after defining the region containing *xer* on the chromosome, this region could be cloned into a vector like pUC8 to allow isolation of its product by biochemical methods. *ckr*$^+$ plasmid DNA could be incubated with *xer* product and the other requirements for standard *in vitro* site-specific recombination systems.

Setting up such an *in vitro* system would allow one to carry out a series of experiments like *in vitro* DNA foot printing experiments to determine the binding site(s) of *xer* product to *ckr* sequence. The reversibility of *ckr* recombination could be checked by incubating monomeric molecules of *ckr*$^+$ plasmid with *xer* product and then analysing the products on agarose gel. The presence of any multimer would mean that the reaction is reversible. Studies on the effect of DNA conformation on recombination between *ckr* copies, would be possible by incubating *ckr*$^+$ supercoiled, nicked-circle, and linear DNA molecules separately with *xer* product, then on an agarose gel the products of the three reactions could be checked.

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